THE NATURE
OF VIRUSES

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CIBA FOUNDATION SYMPOSIUM
ON
THE NATURE OF VIRUSES

Editors for the Ciba Foundation
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With 57 Illustrations



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PREFACE

THE suggestion that one of the Ciba Foundation's small, informal, international conferences should provide an opportunity for discussion of some fundamental aspects of virology originated in Cape Town, in conversations between Professor M. van den Ende and Professor F. G. Young. The latter, as a member of the Council of the Foundation, pursued the matter in detail on his return to England, and the Director readily took up the proposal.

The symposium was arranged for March, 1955, under the title of "The Biophysics and Biochemistry of Viruses". Sir Charles Harington, Director of the National Institute for Medical Research, consented to act as Chairman, and also gave invaluable advice on membership and the construction of the programme. Perhaps even more than on previous occasions, the Director regretted his inability to include in so small a group many of the outstanding contributors in this field of research. Those who could be invited and who honoured us with their presence and contributions were as usual most helpful and co-operative, both in the discussions and in the preparation of this subsequent publication. The Director hopes to be able to invite other virologists on appropriate occasions, and in the meanwhile offers them and other interested workers such participation in this symposium as this volume can give them.

A few explanatory words about the Ciba Foundation may be useful here, though this is the 32nd book containing proceedings of our conferences to be published, and one or more of our other activities may have come to the reader's attention.

The Ciba Foundation is an international centre, established as an educational and scientific charity under the laws of England. It owes its inception and support to its Founder, CIBA Ltd. of Switzerland, but is administered independently and exclusively by its distinguished British Trustees.

The Foundation provides accommodation for scientifi workers who visit London from abroad, organizes and hold international conferences, conducts (in conjunction with th Institut National d'Hygiène) a postgraduate medical ex change scheme between England and France, arranges in formal meetings for discussions, awards two annual lecture ships, has initiated a scheme to encourage basic researcl relevant to the problems of ageing, assists internationa congresses and scientific societies, is building up a librar service in special fields, and generally endeavours to give aid ii all matters that may promote international co-operation ii scientific research.

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G. L. Ada	The Walter and Eliza Hall Institute of Medical Research, Melbourne
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CHAIRMAN'S OPENING REMARKS

Sir Charles Harington

I find my task in opening this symposium a somewhat embarrassing one, since I am the only member of the group who has made no scientific contribution whatever to the subject under discussion; in these circumstances I can speak only as a biochemist who has long been interested in the progress of virus research and who, because of the nature of his job, has attempted to maintain a nodding acquaintance with developments in the field. In this capacity I would like to say first that the most satisfactory feature of the present occasion is that the time has come when it is worth while to hold a symposium within our terms of reference, secondly, may I say how encouraging it is to see gathered here today so many of those to whose efforts the important advances have been due.

It happens that the Institute which I have the honour to direct has been a home of virus research for many years. The history of the work on viruses that has been carried on there goes back indeed to the time when virology had not yet emerged as an independent subject of research and when progress was still inhibited by failure to realize that the elementary biological facts concerning the behaviour of viruses could only be revealed by studies of the infection in the host. We ourselves are proud to remember, as two outstanding events in the scientific history of our Institute, that studies of this kind carried out there led to the major discoveries of the virus of dog distemper and of that of influenza, thus opening up a very wide field of work

Intrinsically important as these biological discoveries were, however, they served themselves to emphasize that for a comprehensive advance in knowledge of the properties of viruses the help of other branches of science would have to be

called in. One of the chief preoccupations of the early workers on viruses was the question of the particle size of the agents that they were studying, and the first real contributions to this problem were provided by the work of Barnard in the development of ultraviolet microscopy and by that of Elford on the preparation and use of filtration membranes of graded porosity. It is indeed remarkable that by this simple method Elford was able to make estimates of particle size so closely in accordance with the values that have been obtained in later years by the use of more complex and accurate physical procedures

The work of Barnard and Elford represents, however, no more than the first elementary attempt to apply the methods of physics to the study of viruses and it leaves untouched the question of their chemistry. The possibility of anything like exact study of the chemistry and physics of viruses had to await the great achievement of Stanley when, in 1936, he succeeded in crystallizing tobacco mosaic virus; this work, with that of Bawden and Pirie and others, immediately made the chemical and physical study of plant viruses an attractive proposition in so far as the material to be studied satisfied at least one criterion of purity and homogeneity. Concurrently the advances in biological methods of cultivation of animal viruses, arising from the egg culture methods of Goodpasture and Burnet and the more recent tissue culture methods developed by Enders and others, have made it possible to obtain these viruses also in what appears to be a homogeneous state, and therefore in a suitable condition for the physicist and chemist to work on them.

What use has been made of these opportunities? Surely a very full one and one that is ever increasing in vigour as we shall learn in the course of this symposium. On the physical side, knowledge of the size and morphology of virus particles has been revolutionized by the skilful application of the high-speed centrifuge and of the electron microscope, the capacities of which have been and are being stretched to the limit in the effort to reveal more details of structure. As for what one

might call the elementary chemistry of viruses, the crystallization of plant viruses has become a commonplace; it is indeed interesting to compare the matter of fact reception of the recent news of the extension of crystallization to the field of animal viruses in the work on poliomyelitis with the intellectual shock that was administered by Stanley's discovery twenty years ago.

Even more impressive is the detailed biochemical analysis of viruses that has been carried out during the last few years and is being so actively pursued today. We have travelled a long way from the mysterious filtrable infective particle of little more than thirty years ago to the present stage when we can envisage a typical virus particle as a structure made up of nucleic acid with a coat of protein. Furthermore, we can distinguish between the two parts of the virus structure, regarding the nucleic acid as the genetic material, whilst the protein coat determines antigenic specificity and provides the mechanisms for attachment of the virus to the host cell and penetration of the genetic material into the cell, presumably also the protein coat carries the specific enzymic properties associated with viruses such as influenza Now we have even the evidence that the protein and nucleic acid portions of certain plant viruses can be dissociated and later recombined to form a reconstituted infective particle, and that, in some cases at least, a partial breakdown of the protein portion is consistent with retention of infectivity.

Clearly discoveries of this sort are providing the basis for an understanding of the host-virus relationship and of the process of virus multiplication. These are not only phenomena of the greatest intrinsic biological interest but they have implications extending far beyond the field of virus research For virus multiplication is after all a special case of protein biosynthesis, and there is no doubt that this general problem will be illuminated by the work that is being done on the structure of viruses and on their chemical composition. In saying this I have in mind such theories of virus protein structure as that developed by Crick on the basis of

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We seem thus to have reached a point at which biochemical and biophysical studies of viruses have really come into their own and offer the greatest prospects of advance. It was this thought that encouraged those who were responsible for arranging this symposium, and they will have their reward if the exchange of views that is to take place here during the next three days does something to accelerate still further the progress that is already so encouraging.

VIRUS STRUCTURE: GENERAL PRINCIPLES

F. H. C CRICK AND J. D. WATSON*

The Medical Research Council Unit for the Study of the Molecular Structure of Biological Systems, Cavendish Laboratory, Cambridge

Introduction

IN this article we shall discuss some general ideas about the structure of viruses. This is a hazardous undertaking. We know of no principles so compelling that we can be certain that they must be true; or, more correctly, those that must be true—the rules for inter-atomic distances, for instance—do not lead directly to any interesting conclusions However, there are certain ideas suggested by experience in related fields (such as the study of protein crystals) which we might well expect to apply to viruses, or at any rate to small viruses. Moreover we can make some use of that powerful but dangerous weapon, the principle of simplicity.

Our ideas fall into two groups. There is good evidence in the case of three plant viruses, and indirect evidence for certain animal viruses, that the protein component of a virus is made up of sub-units. Our first set of ideas concerns the question: why does a virus have protein sub-units? We have not previously published this argument. Our second deals with the problem if there are sub-units, how are they arranged? This we have recently put forward elsewhere, so that we shall only deal with it briefly This paper should therefore be read in conjunction with our previous one (Crick and Watson, 1956).

We shall restrict our discussion in the first place to those small viruses which contain only protein and ribonucleic acid (RNA): that is, the majority of known plant viruses, and

* On leave from the Biology Department, Harvard University, and supported by a grant from the National Science Foundation (U S A)

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certain animal viruses such as poliomyelitis and the various encephalitic viruses.

The reason for protein sub-units

Our basic argument is that the protein component of a virus is unlikely to be either one large molecule or, alternatively, an assembly of small molecules, each of which is quite different from all the others. More precisely, we mean by "different" that the sequence of amino acids in any two such small molecules is quite unrelated.

Our first assumption is that an essential requirement for a virus of this type is that it should consist of a packet of RNA protected by a coat of protein. It is found experimentally that the molecular weight of the RNA is of the order of, say, 2×10^6. Imagine that this amount of RNA is folded as compactly as possible, so that it forms a rather dense sphere. Such a sphere could hardly be less than 150 Å diameter, and is more likely to be nearer 200 Å. We next surround this with a layer of protein, which we shall assume is more or less continuous. There must be a minimum thickness for such a layer; 1 Å, for example, would be impossibly small. A more reasonable minimum value would be 10 Å. Actually no protein crystal is known with a unit cell dimension of less than 24 Å, so that perhaps 20 Å would be a more realistic lower limit. This would require a volume of protein of about 10^7 Å^3, or a molecular weight near 7 million. The details of the calculation are unimportant; the point is that we require a large amount of protein. Notice that the ratio of protein to RNA *increases* as we make the virus smaller; that is, if we had considered a smaller amount of RNA we should not reduce the amount of protein required by very much. We can only have a much smaller *proportion* of protein if the virus is considerably bigger.

The model we have described must not be taken as a detailed model of a virus. It is used purely for illustration. If we follow through the argument for a rod-shaped virus of small diameter we reach a similar conclusion.

Thus, if our assumption that a small virus has to have a reasonably continuous protein coat is correct, we can conclude that a relatively large amount of protein will be required for it. Whatever the reason, the experimental evidence shows clearly that a considerable amount of protein is always present. In Table I we have set out the figures for all the

Table I

AMINO ACID AND NUCLEOTIDE RESIDUES IN RNA VIRUSES

		Molecular Weight	*% RNA*	*% Protein*	*Nucleotides per Particle*	*Amino Acids per Particle*
Tobacco mosaic virus		40×10^6	6	94	7,300	340,000
Potato virus X	$\simeq$	30×10^6	6	94	5,400	260,000
Potato virus Y		75×10^6	5	95	11,300	650,000
Bushy stunt virus		9×10^6	16	84	4,400	69,000
Turnip yellow mosaic virus		5×10^6	40	60	6,000	27,000
Southern bean virus		6×10^6	21	79	3,800	43,000
Tobacco ringspot virus	$\simeq$	6×10^6	40	60	7,300	33,000
Tobacco necrosis virus	$\simeq$	6×10^6	18	82	3,300	43,000
Poliomyelitis		10×10^6	24	76	7,300	69,000
Influenza*		100×10^6	2	75	6,000	680,000
Fowl plague*		100×10^6	2	75	6,000	680,000

The figures in this Table are only approximate

* These viruses may contain material from their host cell

small viruses for which data are available. It can be seen that in every case the *total* number of amino acids always greatly exceeds the *total* number of nucleotides.

Our next assumption is more difficult to justify. It really falls into three parts. We assume (*a*) that the amino acid sequence of the protein component of the progeny is determined wholly, or at least to a large extent, by the infecting virus, (*b*) that this amino acid sequence is determined by the molecular structure of the RNA of the infecting virus, and not at all by its protein component; (*c*) that the "coding" implied in (*b*) is relatively simple.

Of course none of these assumptions is new, though we believe that our argument as a whole is original.

We wish, from these assumptions, to make a crude estimate of how much protein can be "coded" by a given amount of RNA. To fix ideas consider a scheme in which the first three bases of the RNA chain is a code for the first amino acid in the polypeptide chain, the next three bases for the second amino acid, and so on. For an RNA chain of molecular weight 2×10^6, which has about 6,000 bases, this implies that we can code for a polypeptide chain (or chains) of total length 2,000 amino acids, or about 230,000 molecular weight. To form a protein coat, however, we need at least 10 times as much as this, and probably 20 or 30 times as much.

At the moment we know practically nothing about the "code", so we cannot tell whether the estimate of three bases to one amino acid is a good one. However, this ratio, which we have taken as 3 : 1, can scarcely be less that 1 : 1, and a careful study of known amino acid sequences in (non-viral) proteins (Gamow, Rich and Ycas, 1956) suggests that a 1 : 1 ratio is unlikely, since such a code necessarily puts considerable restrictions on the possible amino acid sequences, and these are not apparent.

The way out of this difficulty is obvious. There seems to be no reason why the virus-cell system should not produce a large number of copies of the protein molecules for which the RNA of the virus is a code. The protein coat of the virus would then consist not merely of *one* of these molecules, but of a number of them, and in this way one can obtain a large "molecular weight" from a system which can only produce relatively small protein molecules

We can now summarize our argument in slightly different terms. The information required to synthesize the virus protein is contained in the RNA. As there is only a limited amount of RNA it can only carry a limited amount of information. Thus the protein molecules of the virus can only be of limited size. Rough numerical estimates show that this amount, used once, is not enough to produce a shell to cover the RNA. Thus the coat must be built up of identical sub-units.

The Coding Ratio

If, as we surmise, the "coding ratio"—the number of nucleotides which code, on the average, for one amino acid—is a constant throughout Nature, the plant viruses present very attractive material from which to obtain this ratio experimentally. When we come to grips with the problem, however, we run into formidable difficulties. Only for tobacco mosaic virus (TMV) can we be even approximately sure of the sub-unit size, and here the number of nucleotides in the virus is approximately 50 times the number of amino acids in the protein sub-unit. This figure seems much too large to have a direct bearing on the coding problem. There are several *possible explanations for the large value. One, which we must* consider most seriously, is that the RNA of TMV controls the synthesis of other proteins in addition to that which forms its outer shell Another is that the virus may contain several or many copies of the fundamental RNA chain Still another possibility is that the protein sub-units are not all completely identical, so that the RNA is responsible for the synthesis of several closely related proteins. At first glance bushy stunt virus looks more hopeful, as we find that there are only four nucleotides per amino acid residue in the crystallographic sub-unit This answer, however, is probably misleading, for the chemical data (de Fremery and Knight, 1955; quoted in Caspar, 1956) hints that each crystallographic sub-unit may contain perhaps five chemical sub-sub-units, thus giving 20 nucleotides per amino acid residue, which seems unreasonably large. It is clear that much further work, from many approaches (including that of genetics), will be required before a reliable figure can be obtained.

Experimental Evidence

It would be impossible in a short article to review in detail the evidence in favour of each of our assumptions, but we can mention some of it briefly. We should state straightaway that it is, at the moment, inadequate in almost every case.

The assumption that the RNA is protected by a coat of protein is supported to some extent by the fact that these viruses are not attacked by ribonuclease. The evidence for the protein coat is good for tobacco mosaic virus (TMV) (see Williams, 1956; Franklin, Klug and Holmes, 1956) and for turnip yellow mosaic virus (Markham, 1951; Bernal and Carlisle, 1951; Schmidt, Kaesburg and Beeman, 1954). Even if this assumption is wrong, Table I shows clearly that, whatever the reason, the protein/RNA ratio is large for all the viruses studied.

For our assumption (*a*)—that the amino acid sequence of the progeny virus is determined by the genetic specificity of the infecting virus and not to any significant extent by the host cell—the evidence is again only suggestive. It rests largely on the existence of the mutant virus strains. These strains breed true within the usual plant hosts and have been shown (see Knight, 1956) to possess characteristic differences in their amino acid compositions. However a recent paper (Bawden, 1956) suggests that the host cell may play a far more significant rôle than commonly supposed.

Assumption (*b*)—that the amino acid composition of a virus is determined by the RNA of the infecting virus—is supported by the classic experiment of Harris and Knight (1952; 1955) in which they removed the terminal threonine from the protein sub-units of TMV and found them again in the progeny, and more strongly by the very recent work of Fraenkel-Conrat (1956). In a typical experiment protein from standard TMV was combined with RNA from the Holmes ribgrass strain. After infection the resulting progeny had a protein component closely resembling that of the Holmes ribgrass strain. Notice that it is immaterial to our argument whether the infective unit is RNA alone* or the recombined RNA plus protein. The essential experimental requirement is that no protein from the Holmes ribgrass strain should get into the plant. This experiment of Fraenkel-Conrat's is one of the

* Added in proof: see the important recent paper of Gierer and Schramm (1956) in which it is reported that the RNA alone is infective

utmost importance, not only for virus work, but for the whole field of protein synthesis, and we look forward to it being repeated and extended by other workers* so that its apparent conclusions can be established beyond doubt.

We know of no experimental evidence which directly supports our third postulate (c).

The arrangement of protein sub-units

We have very recently discussed this (Crick and Watson, 1956) in an attempt to answer the question, "Why are all small viruses either rods or spheres?" Here we will put the question the other way round and ask, "Given that the protein component is made of sub-units, how will they be arranged?"

In our view it is not very likely, though not impossible, that the sub-units will aggregate in a random manner On general grounds we would expect that the preferred arrangement will be one in which every sub-unit has the same environment as every other sub-unit This can only be done if the sub-units are related by symmetry elements. The two most likely arrangements are a spherical shell, having cubic symmetry, or a cylindrical shell, having a screw axis, though other symmetries could occur. There are three possible arrangements of symmetry elements which can give cubic symmetry, having 12, 24 and 60 asymmetric sub-units respectively. Thus we predict that many small spherical viruses will have 12n protein sub-units, where n is an integer. Our original article should be consulted for details.

The X-ray evidence has established the existence of sub-units for TMV (see Franklin, Klug and Holmes, 1956), for tomato bushy stunt (Caspar, 1956) and for turnip yellow mosaic (Franklin and co-workers, unpublished). Note that the X-rays can only show that the sub-units are structurally similar, not structurally identical, let alone chemically identical Also note that the crystallographic sub-unit may consist

* Added in proof see the important recent paper of Gierer and Schramm (1956) in which it is reported that the RNA alone is infective

of several chemical sub-units, which may or may not be identical. The X-rays are also incapable of showing whether the sub-units are held together by physical bonds only, or also by chemical bonds, such as S-S bridges, which might be formed during or after the aggregation process.

Very little is known about the detailed arrangement of the RNA in these viruses. It is a reasonable hypothesis that the RNA has the symmetry, or at least some of the symmetry elements, of the protein component. This symmetry may not apply to the precise sequence of the bases of the RNA but only to the phosphate-sugar backbone.

It is rather striking that the absolute content of RNA, for both the rod-shaped and the spherical viruses, varies over rather narrow limits compared with the highly varying amounts of protein. Almost all these small viruses contain between 3,000 and 7,500 nucleotides and we wonder whether there may be an effective lower limit to the size of a virus corresponding to the amount necessary to make a moderately sized protein molecule. There are reports of smaller viruses, in particular the Rothamsted strain of tobacco necrosis virus (Bawden and Pirie, 1950), but there seem to be strong doubts whether these small particles of 150 Å diameter are infective. If they are we should have a virus of $1 \cdot 5 \times 10^6$ molecular weight, containing about 900 nucleotides. According to our above arguments, this amount of RNA might be expected to control the synthesis of a protein of 20–30,000 molecular weight.

The possibility that the *arrangement* of the RNA may be practically the same in all spherical viruses should not be overlooked, since the ways of folding a fibrous molecule so that it has cubic symmetry may be rather limited. For this reason it is quite possible that microsomes, which also appear spherical and are of a similar size to the smaller viruses (actually a little smaller, but the percentage of RNA is probably greater), may also have cubic symmetry, and perhaps possess protein sub-units. In any case, since microsomes contain only a limited amount of RNA, we would predict, by

an extension of our first argument, that no very large protein molecule will be found that is not an aggregate. The fragmentary evidence available on large protein molecules (such as myosin) is compatible with this idea.

Other Viruses

We shall only discuss this very briefly We think it likely [illegible]

ideas may have to be adapted somewhat in special cases, such as the influenza virus, the outer shell of which may not be so well ordered as the inner part and probably consists in part of components of the host. Bacteriophages, such as T2, present a very challenging problem. We feel that our ideas may apply to the separate parts, but even so the shape of the head requires some special explanation.

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DISCUSSION

(*Dr Caspar's remarks were illustrated with a variety of models*)

Caspar · I should like to make some comments to indicate how symmetry can lead to spherical virus particles. First, Dr. Crick has pointed out that you cannot have mirror symmetry in a virus because it is built up of asymmetric molecules. Therefore there are only two types of

operation on a structure—that afterwards it looks the same as it did before. In this case we would say that the wheel has a fourfold rotation axis. Translational symmetry applies to a repeating linear. In bio-

case are rotational symmetry. If there were just one axis of rotational

metry. (The platonic solids actually have mirror planes and a centre of symmetry as well as rotation axes, but for illustration we can ignore these.)

Symmetry does not tell us what a particle is going to look like; it just indicates how the parts are related. We have experimental evidence from X-ray work in the case of bushy stunt virus that it has the symmetry of the regular dodecahedron. Dr. Klug and Mr. Finch have also found that turnip yellow virus probably has this same type of symmetry.

Let us go back to the tetrahedron to indicate how you get sub-units from symmetry. Consider one of the threefold rotation axes. If I arbitrarily put a sub-unit in any place, I have to put another two,

related by this threefold rotation axis, on the same face. Since there are four identical faces I end up with twelve sub-units, arranged in a very definite way. Likewise for the cube or the octahedron I shall end up with twenty-four sub-units. In the dodecahedron there are twelve five-fold faces, so that this will lead to sixty sub-units, arranged in a regular way.

[illegible]

symmetry.

[illegible]

not been this hypothesis,

Williams Dr Crick, is there any particular specification to the coding that makes a coding ratio of something like 1 to 10 and 1 to 20 quite unreasonable?

[illegible]

Watson: The X-ray evidence of Caspar indicated that the protein coat of bushy stunt virus is constructed from 60 similar sub-units. Each sub-unit has a molecular weight around 125,000 and contains approxi-

sequences

argument?

Watson. No, I mean the protein shell is made up of a series of sub-units, and the RNA is sufficient to code to provide the information to

know. In fact, I think the real point of this idea is to bring home

million?

say, for example, that the length of an RNA chain in TMV is only 50 residues, as was once claimed

[illegible]

you might resonably call crystallization.

Knight: Do you think that this perhaps explains why we have some

Watson: The X-ray evidence of Caspar indicated that the protein coat of bushy stunt virus is constructed from 60 similar sub-units. Each sub-unit has a molecular weight around 125,000 and contains approximately 1,100 amino acids. The RNA content of bushy stunt is 16 per cent which corresponds to about 4,400 nucleotides. We thus find four [illegible]

sequences.

Crick: [illegible]

argument?

Watson: No, I mean the protein shell is made up of a series of sub-units, and the RNA is sufficient to code to provide the information to determine the sequence in one of the protein sub-units.

[illegible]

know. In fact, I think the real point of this idea is to bring home [illegible]

expose itself to the whole of one sub-unit?

Crick. What we assume is that the RNA takes some extended form and that the coding is done when the polypeptide chain is this extended form, and that it subsequently folds up. That is the basic answer. How [illegible]

million?

Crick. Perhaps I should say particle weight. We use that figure for illustration because that is approximately what is found It could be that the RNA was in sub-units itself, and the sub-units could be identical or different. However, I would be cautious about arguments that

STRUCTURE AND SUBSTRUCTURE OF VIRUSES AS SEEN UNDER THE ELECTRON MICROSCOPE

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IN recent years the advances in electron microscopy have been such as to allow us to obtain rather precise information regarding the three-dimensional shape of individual virus particles, and reasonably credible notions of the general features of structural differentiation within some of the larger viruses (see reviews by Williams, 1954; Bang, 1955). In addition, the investigation of shapes and sizes of some virus substructures has been initiated, with results so promising as to indicate the desirability of quite intensive investigation along these lines. The electron microscope is unique in furnishing morphological information about individual virus particles by direct optical imagery; other biophysical methods must not only deal with the average properties of multitudes of particles but also yield morphological information quite indirectly.

General appearance of intact virus particles

Only a score or so different viruses have so far been obtained in sufficiently purified form to allow significant electron microscopic observations to be made, but from these observations certain generalizations regarding shape and size may be drawn. The largest of the objects properly called viruses are about 300 mμ. in diameter, while the smallest yet observed are about one-fifteenth that size. Although detailed variations in

chromotrypsinogen is a precursor of chromotrypsin, and it needs some active enzyme to turn X-protein into A-protein, for example, or alter-

finished virus.

Dulbecco: The idea of the crystallization of protein units is useful also to explain some experimental data, like phenotypic mixing of viruses. It is known that the protein coat of a phage particle produced in mixedly infected cells can have variable proportions of the adsorption properties of the two parents, moreover, the composition of the protein, as determined by this property, is independent of the genetic composition of the DNA of the particle. So that it seems that the phage coat is made up of units which are built independently and then assembled together with DNA.

subsequently found to exist for all the T-phages by Williams and Fraser (1953) using a freeze-drying technique. Quite distinct polyhedral figures are found for the particles of tobacco ringspot (Steere, 1956), and less convincingly so for tomato bushy stunt virus (Williams, 1953c) On the other hand, the freeze-drying method does not invariably produce polyhedral structures, since the particles of poliomyelitis virus (Schwerdt, Williams, Stanley, Schaffer and McClain, 1954), and of both components of turnip yellow mosaic virus (Cosentino, Paigen and Steere, 1956) appear invariably to be spherical. The precise form, if any, of the polyhedral objects observed is difficult to determine, and, except for the T-even phages, has been inconclusively inferred only from the shapes of the shadows of the viruses The heads of the T-even phages appear to be hexagonal prisms with bi-pyramidal ends, the other polyhedra seem to have the general outlines of dodecahedra. Only two of the larger viruses, influenza (Williams, 1953c), and the agent of meningopneumonitis (Crocker and Williams, 1955), have been examined by freeze-drying methods. The former appears to be spherical, while the latter is generally spherical with some signs of structural deformation.

It might be anticipated that the electron microscope would be uniquely useful in the detection of whatever details of structure exist upon the surfaces of virus particles. Thus far [illegible] this purpose has proven [illegible] exception of the virus [illegible]es when air-dried show signs of surface nodules in regular array (Williams, 1953c), no surface detail of any apparent significance has been detected upon any virus The reasons for this failure are not clear. The electron microscope has a resolving power sufficient to allow discernment to be made of structural irregularities of the order of 20 Å in size, if sufficient electron contrast is available. So far, contrast can be introduced into the images of intact virus particles only by the enhancement of topographical detail through shadowing methods. The shadowing

non-uniform in size and appear to have a complex structure involving an outer membrane, a less dense peripheral region, and a dense core. The smaller viruses are distinguished, generally, by an electron microscopic appearance of internal homogeneity and of complete uniformity of size and shape. Most of the smaller viruses seem to inhabit the plant world, but the apparent lack of small animal viruses may be due to our inability to separate in identifiable form such small objects from the welter of other similar particles found abundantly in animal tissues (Williams, 1953*a*; Bachrach and Schwerdt, 1954).

Detailed morphology of some viruses

The direct nature of electron microscopic observation carries with it a considerable hazard of misleading information arising from the many artifacts involved in specimen preparation. A most serious danger is that our observations of necessarily limited fields of view will be atypical ; that false impressions will be secured from a few micrographs that are not randomly representative of an entire virus preparation. A second source of misapprehension arises from structural deformations brought about by the dehydration of virus specimens in the course of preparation for electron microscopy. The former type of artifact has been rather thoroughly *eliminated by the use of representative-field techniques* (Sharp, 1949, Backus and Williams, 1950), while the latter artifact may apparently be greatly reduced by the application of one of the techniques developed for the preservation of three-dimensional structure (Anderson, 1951, Williams, 1953*b*).

The application of the techniques of preservation of three-dimensional structure has given us new information about the precise morphology of a few viruses in the intact form. The most striking and unanticipated finding is that some of the viruses, heretofore considered to be spherical, are in fact polyhedral in shape. This kind of shape was first found by Anderson (1951) for the T-even bacteriophages and was

When frozen-dried, the particles of both components look alike in shadowed preparations. Particles of the medium-sized and smaller viruses, such as influenza, rabbit papilloma, equine encephalomyelitis, tomato bushy stunt, and poliomyelitis (to name a few examples) when observed in unshadowed preparations show no degree of differential electron opacity sufficient to suggest the existence of internal structure. Such uniformity in the electron image is to be anticipated (Hall, 1955) in consideration of the small variations in mass-thickness that would be expected to exist in a biological substance, such as a virus.

Appearance of viruses seen in sections

Considerable success has been attained in recent years in the demonstration of virus particles within thin sections of infected cells. In such sections the particles of the larger viruses are transected, and electron micrographs of the sections show evidence of structural differentiation within the viruses So far, only the larger viruses have been identified sufficiently well in sectioned cells to allow generalizations to be made, but certainly in the case of meningopneumonitis (Gaylord, 1954), fibroma (Bernhard, Bauer, Harel and Oberling, 1955), vaccinia (Morgan, Ellison, Rose and Moore, 1954*a*), and herpes simplex (Morgan *et al.*, 1954*b*) there is a consistent structural pattern. Each virus particle appears to have a relatively opaque central core, surrounded by a shell of transparent material, which in turn is surrounded by one, or two, concentric membranes. Recently it has been found by Bernstein (unpublished) that the heads of purified T2 phages, when stained with iron, show this same general appearance. Unfortunately, it is difficult to assess the meaning of the differences in opacity seen in the electron micrographs. It is not known whether the stain almost universally employed (osmium tetroxide) attaches to all or any portion of the virus, and consequently it is uncertain whether the relatively opaque core arises from a denser material in that

metal itself obviously tends toward obscuration of the finest detail; there is the further suspicion that the surfaces of all objects are coated with tiny oil globules during normal shadowing (Williams, 1952), thus causing the surfaces of viruses to have a general "pebbly" appearance. Another cause of obscuration of surface detail may well be the fine-scale deformations incident to both air-drying and freeze-drying of virus particles. A further cause of the generally nondescript appearance of surface detail is the obvious one that virus particles may simply have no periodic arrangement of surface irregularities. It might be added parenthetically that it is fairly easy to secure electron micrographs which seem to exhibit periodic structure upon the surfaces of virus particles; all that is needed is an inferior micrograph which combines under-focus with either an astigmatic or a moving image.

Some evidences of internal virus structure have been obtained from the electron microscopy of intact virus particles, observed either shadowed or unshadowed. The large viruses of the psittacosis group, and of the herpes group, exhibit a "derby-hat" appearance after dehydration from an aqueous suspension. Such differences of rigidity clearly imply a corresponding internal structure, with a relatively undeformable central core and an outer membrane, but the morphological observations alone give no clue as to the composition of the core. More precise information has been obtained for members of the pox group of viruses, where treatment with enzymes clearly indicates that the central core is composed in good part of nucleic acid (Dawson and McFarlane, 1948; Peters and Nasemann, 1952; Peters and Stoeckenius, 1954). The existence of a central core of some nature within the infectious component of turnip yellow mosaic virus, and its absence in the non-infectious component, has recently been demonstrated in electron micrographs by Cosentino *et al.* (1956). In this case use was made of drying artifacts to show that the particles of the non-infectious component have, when dried, a "dimple" in their centres.

the planing of the ice block, but no high resolution micrographs have yet been obtained.

Virus substructures

As might be anticipated, the electron microscope is proving uniquely useful in the visualization of the products of virus disintegration and re-assembly, especially in those cases where some sort of biological or chemical activity can be associated with morphologically distinctive components of the disintegrated particles. An example of this sort of application is found in studies made on the biological activity of certain small particles obtained from the disintegration of the fowl plague virus (Schafer and Zillig, 1954). It was found that two distinct, soluble components could be released from this virus, one of which has antigenic and immunizing properties, and the other of which has red cell agglutinating properties as well. Electron micrographs show the two types of particles to have very small, but distinctive, sizes; unfortunately, the particles are so small and presumably so affected by drying that their quasi-spherical appearance may well have no special significance.

Bacteriophage

Two viruses whose disintegration has been rather thoroughly studied by electron microscopy are T2 bacteriophage and tobacco mosaic virus. Fraser and Williams (1953) were

material was in the form of distinct strands, rather thoroughly intertwined, the diameter of which was about 20 Å. The strand length could not be determined owing to the intricate coiling of the material, but it was evident from the very few free ends seen that the individual strands of DNA were at least several hundred Å long. Kellenberger and Arber (1955), and Williams and Fraser (1956), have examined the structure of

portion of the virus or whether it arises from the osmophilia of that region. There can be no denying, however, that the "larger" viruses (i.e., those larger than influenza) show quite enough evidence of the structure described above to cause virologists to believe in its reality.

Steere (unpublished) has recently developed a technique which is applicable for the detection of some types of structural differentiation within viruses. In this method the material, such as a virus suspension, a crystal, or a virus-infected cell, is fixed by quick freezing. Part of the frozen block is planed away to produce a smooth surface. This surface is then sublimed *in vacuo* to a depth of about 100 Å. Surface detail will arise during the sublimation if there is a differential sublimation rate between, say, a virus particle and the surrounding ice. A shadowed replica is made of the sublimed surface, and is then removed from the surface and examined in the electron microscope. Only two viruses have so far been examined in this manner, tobacco ringspot in the form of crystalline material, and tobacco mosaic virus in the form of natural inclusion crystals in hair cells. The former virus exhibits, apparently, space-filling properties of packing, with each virus particle showing pronounced regularity of outline in transection. No detail of structure is seen over the cut surfaces of the particles; perhaps one should not expect the relative rates of sublimation of water hydrated with protein and with nucleic acid to be different. It will be of great interest to observe mixed crystals of turnip yellow mosaic virus by this method of sublimation-replication. The inclusion crystals of tobacco mosaic virus exhibit a considerable degree of regularity of orientation of the included virus particles, as might be expected. The particles appear stacked in plates, with the individual rods not exactly perpendicular to the faces of the plates. The arrangement of the rods in the plates, and of the plates with respect to each other, is apparently that deduced by Wilkins, Stokes, Seeds and Oster (1950). So far there has been no detail of structure apparent within those virus rods which have been transected during

presumably protein structures There is some evidence that at least the distal half of the tail sheath is composed of a helical wrapping. Since it seems feasible to separate and purify the tail fibres in small quantities, it would appear that here is a species of fibrous macromolecule of some interest, possessed as it is of a distinctive type of biological specificity.

Tobacco mosaic virus

The apparent regularity of the structure of tobacco mosaic virus (TMV), as contrasted with the complexity of structure of the bacteriophages, makes it seem most likely that electron microscopic observations of its substructures will have considerable relevance in relation to structural information gleaned from other types of inquiry. As mentioned previously, even the highest resolution micrographs of the particles of the intact virus are disappointingly lacking in information; all that can be discerned is a straight rod of indeterminate cross-sectional shape and exhibiting no regularity of detail in its surface structure Some years ago an attempt was made to see if the cross-sectional shape could be inferred from observations of very short fragments of the virus rods produced by sonic oscillation (Williams, 1952) The observations, subsequently confirmed informally by Hall and by Kaesberg (personal communications), indicated that these short fragments are frequently seen in cross-section as fairly regular hexagonal platelets. From these appearances it was concluded that a most likely form for the intact particle was that of an elongated hexagonal prism The X-ray observations, however, (Watson, 1954; Franklin, 1955) conclusively show a helical structure for the protein of the virus rods, and the internal packing appears to be such as to make an overall hexagonal contour unlikely. We are now left with the dilemma as to why short portions of the rods should exhibit hexagonal outlines despite the more intricate external contour predicted for the entire particle by the X-ray results.

As Schramm (1947) has shown, it is possible to disintegrate TMV by treating it at high pH and to recover the protein

the components of disintegrated T-even phages, with particular attention given to the tail structure. Disintegration was accomplished by treatment with oxidizing agents (Kellenberger and Arber) and by freezing and thawing (Williams and Fraser). The effect of the oxidizing agents (such as H_2O_2-alcohol) is to solubilize a portion of the distal end of the tail, leaving an intact, inner core and a small number of slender fibres at the very tip of the core. This disintegration of the tail sheath takes place in only its distal half. The oxidized tail structures are still capable of adsorption to host cells, but only in a reversible manner. The discovery that the tail sheath is more resistant to disintegration by oxidation in its proximal half reminds one of the observation by Levinthal and Fisher (1953) that phage particles, sheared off their host cells after prolonged adsorption, exhibit tails of only half length.

Williams and Fraser (1956) were able to make quantitative observations of the adsorbing proclivities of the components of disjoined T2 phages by application of particle-counting techniques (Backus and Williams, 1950). They confirmed the previous indications that separated phage heads are incapable of adsorption. The head structure itself is frequently left quite intact, and filled with DNA, despite the apparent removal of the entire tail. The separated tails are able to adsorb just as efficiently as they do when part of the intact phage. The freezing and thawing accomplishes a partial, or even complete, disintegration of the tail sheath, occasionally uncovering a stiff core of length equal to that of the entire tail. Although the evidence is incomplete, it is believed that the naked cores do not adsorb to host cells. The tail fibres may be separated from the tails, and appear to have a distinctive size and shape. They are about 6 mμ. in diameter and usually appear in the form of an obtuse V, the total length of which is quite constantly 130 mμ. The V frequently breaks at the tip, as evidenced by the frequent occurrence of straight fibres some 65 mμ. long. The separated tail fibres are capable of complete adsorption to host cells. Neither the core nor the tail fibres are attacked by ribonuclease or desoxyribonuclease, and are

treated at high pH. In neither case, however, was the identification of the threads with RNA buttressed by any sort of chemical test.

Hart (1955) has developed a technique whereby TMV may be very mildly and controllably degraded with sodium dodecyl sulphate at elevated temperature and immediately dried for examination in the electron microscope. This treatment produces rods whose ends show signs of degradation, in that they are seen to have protruding from them a core material of about 50 Å diameter. The core appears to be axially localized where it joins the residual intact rod. Treatment with RNase causes the core material to disappear, but treatment with DNase or with trypsin has no effect From these experiments it would appear that the core is RNA and that it is axially localized in the intact virus In general the protruding RNA shows no sign of fraying into thinner fibrils. The electron micrographs do not, as yet, appear to offer any answer as to whether or not the RNA is arrayed around a true hole on the axis of the virus rod, and whether or not the RNA is single or multiple stranded.

Re-assembly of tobacco mosaic virus

The re-aggregative proclivities of the protein obtained from tobacco mosaic virus by treatment at high pH were shown by Schramm (1947) It was found that a lowering of the pH produced a type of re-assembly of the protein sub-units (the "A-protein") The aggregated material gave ultracentrifuge patterns indicating the presence of particles like those of native TMV, but the particles were devoid of infectivity. That this tendency towards regular aggregation is not restricted to the protein derived from TMV was shown by Takahashi and Ishii (1953), since they were able to cause an abnormal protein extractable from infected plants (the X-protein) to aggregate into rods, shown by electron microscopy to be morphologically indistinguishable from native TMV. As might be expected, the aggregated X-protein is not infectious. It should be noted that, although the rods

portion in an apparently native form. This material is mostly in the form of particles of molecular weight ~100,000, too small to be discerned with any morphological significance in the electron microscope. Not all the protein is reduced to this size, however, and furthermore, the 100,000 mole wt. units readily aggregate into somewhat larger structures. Consequently, when the protein fraction is observed under the electron microscope, some particles are seen whose size is sufficient to disclose a distinctive shape—that of an approximate disc with a central hole, like a machinist's washer (Schramm, Schumacher and Zillig, 1955; Fraenkel-Conrat and Williams, 1955). The central hole has a diameter estimated to be 50 Å, and inasmuch as the protein material is known to be devoid of RNA, it is reasonable to suppose that the hole represents the space occupied by the RNA in the intact virus.

The nucleic acid fraction of TMV can be obtained in apparently undegraded form by treatment of the intact virus with detergent (Fraenkel-Conrat and Singer, 1954). Attempts in this laboratory to photograph the RNA so prepared have been quite frustrating. Although it is clear that some kind of stranded material is being observed, there is no distinctive type of fibre seen; rather, there appear to be gradations of fibre widths all the way from barely discernible threads to [illegible] material [illegible] ints, and [illegible] solution is lost during drying.

Despite the disappointing appearance of the purified RNA from tobacco mosaic virus, it has proved possible to demonstrate its localization within almost intact virus rods. Intimations of its localization have been presented by Stahmann and Kaesberg (1955), and by Schramm, Schumacher and Zillig, (1955). In the former demonstration it was shown that occasional particles of frozen-dried TMV are fragmented

nor has an active preparation failed to show them in appreciable numbers.

By the use of Hart's technique it has been shown that the reconstituted material contains its moiety of RNA in the form of a central core (Fraenkel-Conrat and Williams, 1955). The only discernible distinction in this regard between the two kinds of TMV, native and reconstituted, is that the latter is distinctly more labile to the effects of the detergent and elevated temperature used to disclose the inner core.

Recently it has proved possible (Fraenkel-Conrat, 1956) to reconstitute what might be called a mixed, or hybrid, virus

infectivity, and the progeny viruses are found to possess the strain characteristics of the virus which furnished the nucleic acid. It is perhaps unnecessary to add that the electron microscope shows the reconstituted material to be identical (except for length distribution) to native TMV.

No investigation has yet been made of the kinetics of the reconstitution reaction by following the change of length distribution as a function of time following the mixing together of the two components. At the concentrations so far employed the reaction is physically completed in a very few minutes, and it is likely that we have always examined the products after the physical reaction is complete. It does seem a little surprising that we have never observed any incompletely formed particles, in the sense of particles that are fully formed throughout only a portion of their length with a bit of RNA left over at one end

It now begins to appear (Fraenkel-Conrat, 1956) that infection can be obtained from solutions in which essentially no full-length TMV particles, either native or reconstituted, are present. The active solutions are believed to be pure RNA, and are infectious when rubbed upon tobacco plants in sufficiently high concentrations. The same solutions, when assayed at the much lower concentrations employed in the tests for reconstituted activity, show no infectivity. Electron

obtained from the aggregation of both the A-protein and the X-protein have the same diameter and general appearance of TMV, they do not possess the monodispersity of length exhibited by the latter (Williams and Steere, 1951). There appears to be a morphological identity between slightly aggregated X-protein (with particle weights around 10^6) and particles of similar size of partially aggregated A-protein; that is, they both appear as discs with central holes.

Schramm and Zillig (1955) have recently shown electron micrographs which they interpret as indicating that the rods of aggregated A-protein exhibit a periodicity of transverse surface structure. Electron micrographs obtained in this laboratory do not support this conclusion, but rather, show the same sort of pebbly structure upon the surface of the A-protein as is seen on the surface of native TMV particles, aggregated X-protein, and re-constituted particles of TMV nucleoprotein (to be discussed below).

It was found by Fraenkel-Conrat and Williams (1955) that the A-protein derived from tobacco mosaic virus and the RNA obtained by treatment of the virus with sodium dodecyl sulphate will combine stoichiometrically at a pH above that at which the A-protein alone will aggregate to form rods. The re-combined nucleoprotein contains about 6 per cent RNA, as does the native virus. The resulting rods are identical in appearance with the virus, except that there is a large range of distribution in length. Most of the rods are less than 3,000 Å long, some are precisely that length, while a few are longer. It is difficult to set precise figures to these estimates, because the length distribution varies from one preparation to another. Fraenkel-Conrat and Williams also found that the reconstituted rods, from non-infective component materials, have an infectivity, varying from preparation to preparation, but sometimes amounting to about 2 per cent of that of the native virus. Although the reconstituted material is heterodisperse in length, there is a high degree of correlation between the presence of rods of length $\geqslant$ 3000 Å and infectivity; at no time has an inactive preparation shown rods of this length,

nor has an active preparation failed to show them in appreciable numbers.

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Recently it has proved possible (Fraenkel-Conrat, 1956) to reconstitute what might be called a mixed, or hybrid, virus. It is found that the protein of wild-type TMV will recombine with the RNA of Holmes ribgrass. This combination has some infectivity, and the progeny viruses are found to possess the strain characteristics of the virus which furnished the nucleic acid. It is perhaps unnecessary to add that the electron microscope shows the reconstituted material to be identical (except for length distribution) to native TMV

No investigation has yet been made of the kinetics of the reconstitution reaction by following the change of length distribution as a function of time following the mixing together of the two components. At the concentrations so far employed the reaction is physically completed in a very few minutes, and it is likely that we have always examined the products after the physical reaction is complete It does seem a little surprising that we have never observed any incompletely formed particles, in the sense of particles that are fully formed throughout only a portion of their length with a bit of RNA left over at one end

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micrographs of solutions of RNA which prove to be infectious, and those which do not, are unfortunately identical in appearance and are quite uninformative. In no case, however, do they disclose the presence of any rods of length approaching 3000 Å.

The results of electron microscopic investigations of the structure of tobacco mosaic virus appear to be quite generally consistent with the X-ray and chemical evidence. It would seem that there is a central core of RNA, perhaps in itself hollow, around which is wrapped in helical fashion the identical protein sub-units. Either the grooves in the virus surface, postulated by Franklin (1955) are quite shallow, or else the conditions leading to the electron microscopic examination are such as to obscure the visualization of these grooves. For infection, it appears that either an intact virus rod (i.e., one containing its full complement of length of RNA) is necessary, or else that the RNA fraction alone must be in sufficient concentration to guarantee that a minimal number of fully intact RNA "units" are present. Whether or not *pure* RNA is in itself infectious remains to be seen; all that can be said now is that *highly purified* RNA appears to be so, and that, at any rate, solutions devoid of full-length TMV rods can carry infectivity if tested at sufficient concentration.

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DISCUSSION

Pirie Dr Williams, what is the physical form of the ring spot pre-

allows an analysis of the morphogenesis of the phage. Certainly the nucleic acid [illegible]

is [illegible]

als[illegible]

[illegible] as shown by F. Jacob and E. Wollman, the genetic material of the phage is reproduced, phage heads are formed, and some protein analogous to the tail s-protein is produced. But the phage particles are not organized and we know that [illegible] to one defective gene [illegible] group One has, of course [illegible] it seems as though one [illegible] organization of the phage particle.

Crick What your results really show is not that one gene is responsible, but that one gene is indispensable.

Lwoff Yes

Crick. What is the current feeling concerning [illegible]

[illegible] is not known.

Pirie. Dr Williams, does the infective nucleic acid become any more stable if instead of diluting it with water you dilute with reducing agents [illegible]

excess of carrier of RNA that had been shown not to have any activity.

opposite half-leaves of the same plants

One knows in an experiment how many virus rods of TMV/ml will

... and one

titre of native TMV by a factor of 20,000 or so, changes the infectivity of the purified RNA preparation not appreciably.

In the electron microscope, we can observe the RNA at a concentration such that, if TMV particles of length about 3000 Å were present, we would be able to detect them at approximately 10^4 to 5×10^4 particles per ml. With the TMV and the test plants which we use, after making the necessary arithmetical correction to account for dilutions, we are able to obtain infectivity of the amount we would obtain, from 5×10^9 particles per ml, if only TMV were present. So that in something which is demonstrated by the electron microscope to be devoid of TMV particles, at any rate above the 5×10^4/ml. level, we get an infectivity that could be due to contaminant TMV only if at least approximately 5×10^9 particles per ml were present. It looks as if, within a safety factor of about 100 to 10,000, we do not have enough physical TMV particles to account for the lesions observed with the RNA preparation.

Pirie. What do you call a particle?

... length

would be about 200 or 300 Å.

Pirie But we think they can be infective.

Williams: If you are sure, this is a very interesting statement.

Pirie: Now the other point that puzzles me about your observations ... to get a

infective?

that were not infectious, as sometimes occurs, and we have never found

protein.

Whether it needs still to be rods 300 mμ. long I don't know, but perhaps not as it remains in the supernatant fluid after ultracentrifugation However, neither Pirie nor I have ever maintained that particles of any critical length were necessary for infectivity.

Fraenkel-Conrat and Williams failed to note that the protein inhibits infectivity because the test they made, mixing concentrated virus and protein and then diluting the mixture before testing, was the wrong type. They should have tested the effect of concentrated protein on dilute *virus preparations.*

X-RAY DIFFRACTION STUDIES OF THE *STRUCTURE AND MORPHOLOGY OF* TOBACCO MOSAIC VIRUS

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Introduction

THE X-ray diffraction diagram of any substance is a pattern directly related to the structural regularity within the substance In general, the higher the degree of order in the structure investigated, the greater will be the amount of information contained in its X-ray diagram. The scale of the structure which can be investigated by this method ranges from atomic dimensions to a few tens or hundreds of Ångströms.

The earliest X-ray diffraction pictures of plant viruses (Bernal and Fankuchen, 1941; Carlisle and Dornberger, 1948; *Bernal and Carlisle, 1948) showed them to have a very highly* ordered internal structure In recent years, great progress has been made in the study both of the chemical constitution of plant viruses and of the molecular structure of proteins and nucleic acids in general. This, together with advances in the techniques—both experimental and interpretive—of X-ray diffraction, makes it appear probable that intensive study of the X-ray diffraction patterns of plant viruses could now lead to detailed knowledge of the molecular structure of both the protein and the ribonucleic acid in the intact virus particle, and the structural relationship of the one to the other.

This paper describes the early stages of such a study which is being carried out on tobacco mosaic virus (TMV). This virus was chosen both because it is that which has been most extensively studied by chemical and physicochemical methods, and because it is readily obtained in the form of

orientated gel preparations (Bernal and Fankuchen, 1941) which give excellent X-ray diffraction diagrams.

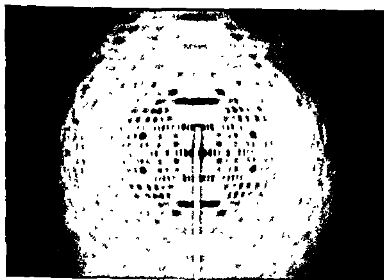
An X-ray diffraction photograph obtained from an orientated gel of a normal strain of TMV is shown in Fig. 1. It is obvious that the amount of information contained in such a photograph is very large indeed. There is, however, no standard or direct method by which this information can be extracted. Some progress towards an interpretation of such photographs can be made by subjecting them to a detailed comparison with photographs obtained from related substances, such as chemically modified TMV preparations or other strains of the virus. It is shown below that such comparisons have already provided some new information concerning the morphology of the TMV particle, the helical arrangement of the sub-units of which the virus protein is built up, and the location of the ribonucleic acid (RNA) in the particle.

Protein sub-units in tobacco mosaic virus

End-group analyses of TMV protein, both at Berkeley and Tubingen, indicate that the protein is built up of sub-units of molecular weight 17,000–18,000 (Harris and Knight, 1952, 1955; Schramm and Braunitzer, 1953; Braunitzer, 1954; Schramm, Braunitzer and Schneider, 1954; Niu and Fraenkel-Conrat, 1955*a*, *b*). The cysteine content of the virus protein also gives a minimum molecular weight of about this value for [illegible]

T [illegible] orientate [illegible] rdered repeating structure within the virus particle. It is clearly of interest to determine the relationship between the structural sub-units indicated by X-rays and the chemical sub-units of the protein.

Watson (1954) showed, by means of X-ray diffraction, that the structural sub-units are set in helical array about the long axis of the virus particle, and that in the axial repeat period of 69 Å there are $3n + 1$ such sub-units distributed over three



[illegible]

facing page 40

turns of the helix. There has been considerable uncertainty as to the value of n. Watson suggested that the value of n was 10. More detailed measurements by Franklin (1955*a*) confirmed that the structure was helical and had $3n + 1$ sub-units on three turns of the helix, but indicated that n might be 12 rather than 10.

We now believe both the above estimates of the value of n to be false. We shall therefore briefly explain how they arose, and why the more recent estimate of 16 for n is considered much more reliable.

If there are u ($= 3n + 1$) sub-units on three turns of the helix, then a diffraction maximum may be expected to occur in the axial direction on the u^{th} layer-line, but on no layer-line lower than this. Watson observed a strong and apparently axial maximum on the 31st layer-line, and hence concluded that u was 31 (i.e. $n = 10$) X-ray diagrams of better orientated TMV preparations (Franklin, 1955*a*) showed, *however, that this maximum lies well off the axial direction.* It can therefore give no information as to the number of sub-units. A maximum on the 37th layer-line, lying *apparently* on the axis, indicated that n might be 12 However, the orientation of the preparation was still not good enough for it to be established with certainty whether or not this maximum was truly axial

When a search was made on the equator and low layer-lines of the X-ray diagram for evidence which might corroborate either of the above estimates of u, no such evidence was found. A fresh approach to the problem was therefore made.

For this purpose, a mercury substituted TMV prepared by Dr. H. Fraenkel-Conrat, was used. In this preparation, mercury is bound to the sulphur of the cysteine residue of the virus protein, in the form $-Hg-CH_3$, to the extent of one mercury atom to about 20,000 molecular weight of virus. Since there is only one cysteine residue in each chemical sub-unit of virus protein, and since the amount of mercury bound is only slightly less than one atom per chemical

sub-unit, the distribution of the mercury atoms in the virus particle will be directly related to that of the chemical sub-units.

From a detailed, quantitative comparison of the X-ray diffraction diagrams of the mercury-substituted and normal TMV preparations it was possible, in effect, to determine the X-ray diffraction diagram of the mercury in the mercury-substituted preparation From this it could be deduced, first, that all (or nearly all) the substituted mercury lay at a radial distance of 57·0 ± 1·0 Å from the axis of the virus particle and, second, that in the axial repeat period of 69 Å the mercury atoms lay in 49 positions equally spaced along three turns of a helix (the radius of the helix being 57 Å). Independent evidence of this arrangement of the mercury atoms was obtained from five different parts of the X-ray diagram.

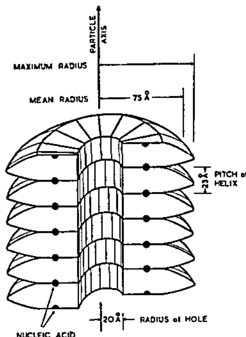
Since there is only one mercury site on each chemical sub-unit of protein, we must conclude that there are 49 *chemical* sub-units on 3 turns of the helix

Returning to the X-ray diffraction diagram of normal TMV, we find that a self-consistent interpretation of much of the diagram is obtained if it be supposed that there are 49 *structural* sub-units in 3 turns of the helix. There is thus strong evidence that the sub-units determined by chemical methods on the one hand and by diffraction measurements on the other are identical.

The helical arrangement of protein sub-units is shown schematically in Fig. 2 (Other features of this diagram are discussed in later sections)

It remains to consider the implications of the above conclusions with regard to the relationship between the molecular weights of the sub-unit and of the complete virus particle. Taking the particle length to be 3000 Å, there are $49 \times \frac{3000}{69} = 2130$ sub-units in the complete helix. If the molecular weight of a sub-unit is 17,000, this gives a total molecular weight of $36 \cdot 2 \times 10^6$ for the protein or, assuming a nucleic

acid content of 6 per cent (Knight, 1954), a molecular weight of $38 \cdot 4 \times 10^6$ for the virus particle. If the sub-unit weight is 18,000, the particle weight calculated in this way is $40 \cdot 7 \times 10^6$.



[illegible] helix), the helical groove and its accompanying helical ridge extending beyond the *mean* radius of the particle, and the hollow axial core The serrated character of the helical ridge is not shown. The nucleic acid is shown at a radius of 40 Å

Our results therefore suggest that the molecular weight of TMV is about 40×10^6. This is in good agreement with the values obtained by Schramm and Bergold (1947) using

sedimentation and diffusion measurements, and by Oster, Doty and Zimm (1947) and Oster (1950) using light-scattering measurements. On the other hand Williams, Backus and Steere (1951), using a direct method involving weighing and electron microscope measurements, found the particle weight to be 50×10^6.

We shall not attempt here to decide between these conflicting values. We wish to stress, however, that, as far as the chemical and X-ray determination of the particle weight of TMV is concerned, the value, 40×10^6, arrived at above refers to the RNA together with the protein sub-units which follow the helical arrangement described. On the basis of the X-ray data alone we cannot exclude the possibility that the virus particle contains *small* amounts of protein organized in a different way.

It is tempting to try to check the correctness of our helical parameters, and of the rather low particle weight which they seem to indicate, by comparing the measured density of TMV with that calculated from the model described here. However, we now know (Franklin and Klug, 1956) (see below) that the particle has not a simple, cylindrical surface, and the calculated density is too sensitive to the mean particle radius assumed, to be of any great value. If we take the mean particle radius to be 75 Å and the radius of the central hole to be 20 Å (see below), the calculated density is 1·30 g./ml., in good agreement with the measured value, 1·305 g./ml. (J. T. Finch, unpublished). (The higher value, 1·37 g./ml. obtained for the reciprocal of the partial specific volume, is probably due to adsorption of water by the virus.) If, on the other hand, we take the mean radius to be 77 Å (Franklin and Klug, 1956) the calculated density, 1·22 g./ml. is too low.

Morphology

It has been shown above that TMV protein is in the form of sub-units set in helical array about the particle axis. It is hard to imagine these protein sub-units being of such a shape that, when helically linked, they would completely fill all

space (except that required for the nucleic acid) in a perfectly cylindrical particle. In fact, X-ray diffraction measurements indicate both that the virus particles are hollow, and that, at the outside of the particle also, the sub-units are not a "perfect fit". These points are discussed below.

The radial density distribution in the TMV particle can be calculated from the equatorial diffraction data if the signs of the diffraction maxima are known. The signs can be determined by the use of a heavy-atom derivative of the virus.

This has been done by Caspar (1956) using a lead derivative, and by Franklin and Holmes using the mercury preparation described above. The result obtained by Franklin and Holmes is shown in Fig 3 (full-line curve). Since the measurements are made on gels of TMV in water, the density values obtained represent the amount by which the electron density of the virus exceeds that of water. In Fig. 3 this is plotted as a function of radial distance from the particle axis. It is clear that the particle is hollow (or water filled) out to a radius of about 20 Å.

Bernal and Fankuchen (1941) found that the inter-particle distance in dry orientated tobacco mosaic virus preparations was 152 Å, and this value has subsequently been accepted as the particle diameter. Inter-particle distances measured by the electron microscope agree well with this figure. It is clear from Fig. 8, however, that the mean particle density remains high out to distances of more than 80 Å from the particle axis, there being a strong density maximum at about 78 Å. It follows that the *maximum* radius of the particle must exceed its
lier by
he 3rd
while
the *mean* radius of the particle was 77 Å, the particle was rather deeply furrowed by a helical groove following the helical arrangement of the protein sub-units. Further, there was evidence that the ridge defining the helical groove was serrated rather than continuous. This suggested that the

ridge could be considered as a helical array of knobs, one knob for each sub-unit.

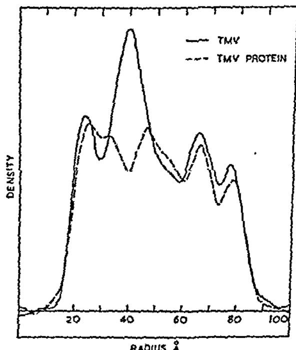


FIG 3 The radial density distribution in
(a) tobacco mosaic virus (full curve), and
(b) repolymerized, nucleic acid-free, tobacco mosaic virus protein (dotted curve) (cf. Franklin, 1950).

These features of the shape of the protein sub-unit are indicated schematically in Fig. 2, which shows a short length of the virus particle cut in half along a plane through the particle axis. (The serrated character of the helical ridge is not shown in the Figure.)

The nucleic acid

No mention has so far been made of the ribonucleic acid, which is present in TMV to the extent of about 6 per cent (Knight, 1954). We shall now discuss some information concerning its location and molecular arrangement in the virus particle, derived from a comparison of the X-ray diffraction diagrams of TMV and of repolymerized, RNA-free, TMV protein

When TMV is treated with dilute alkali (pH 10·5) (Schramm, 1947) the protein is broken down to a molecular weight of about 100,000. The nucleic acid can then be separated by electrophoresis. On lowering the pH, the protein repolymerizes to form rods which, in the electron microscope, closely resemble the virus particles except for a more random distribution of particle lengths. X-ray diffraction diagrams of repolymerized protein prepared in this way by Professor G. Schramm have been obtained by Franklin (1955*b*). They bear sufficient resemblance to the X-ray diagrams of the intact virus to show that the structural arrangement of the protein must be essentially the same in the two materials. They also show important differences, however, and these are being systematically investigated.

From the equatorial scattering of the nucleic acid-free protein, the radial density distribution in the repolymerized particle has been calculated (Franklin, 1956). This is shown in Fig. 8 (dotted curve) where it is compared with the density distribution in the intact virus. It is clear that the principal difference between the two curves is that the very prominent density maximum at a radius of 40 Å in the virus is replaced by a density *minimum* in the nucleic acid-free protein. We can only conclude that the nucleic acid is responsible for this density peak in the virus. Since the phosphate groups in nucleic acid act effectively as "heavy atoms" in X-ray diffraction, it is the phosphate-sugar back-bone chain of the nucleic acid which lies at a radial distance of 40 Å.

In view of the observation by electron microscopists that the nucleic acid appears to form a central core in the virus

particle (Schramm, Schumacher and Zillig, 1955*a*, *b*; Hart, 1955*a*) this result is somewhat surprising. However, a study of diffraction differences between the virus and the nucleic acid-free protein in the non-equatorial regions of the X-ray diagrams provides very ample confirmation that the structural difference between the two substances lies mainly at a radius of about 40 Å.

It will be noted that the density minimum at 40 Å in the nucleic acid-free virus protein is no broader and no deeper than the minima at 60 Å and 78 Å. It seems, therefore, that the nucleic acid must be fitted in a very compact way into the structure of the virus protein.

Since the nucleic acid is both deeply embedded in the virus protein and rather tightly fitted into it, the chain direction of the nucleic acid molecule must clearly be directly related to the helical arrangement of the protein sub-units. Owing to the small amount of nucleic acid in the virus, and to the relatively large radius on which it lies, there is only a very small number (2 or 3) of ways of forming a regular structure for the nucleic acid complying with this condition. Much further work remains to be done to determine with certainty which of the few possible paths the RNA molecule follows. However, it should perhaps be mentioned at this stage, because of its relevance to electron microscope and other studies of the virus, that the arrangement favoured by the measurements made so far is that in which a single nucleic acid molecule follows the line of the main protein helix (pitch 23 Å) throughout the length of the virus particle. (The other possible arrangements involve a number of nucleic acid strands following helical paths inclined at a smaller angle to the particle axis.)

It has been shown by Hart (1955*b*) that, not only can a part of the protein be removed from the TMV particle while leaving the RNA apparently intact and preserving infectivity, but also that the RNA exposed by this treatment is covered up again when more TMV protein is supplied. This suggests that the RNA molecule does not run through the middle of

individual protein sub-units, but rather that the sub-units pack together in such a way as to leave space for the RNA between them.

Little is known concerning the structure of the RNA molecule within the virus particle. Comparison of the birefringence of TMV with that of repolymerized TMV protein (Franklin, 1955*b*) indicates that the base groups are more nearly parallel than perpendicular to the particle axis, and this is in accord with the ultraviolet dichroism measurements of Seeds and Wilkins (1950) and Perutz, Jope and Barer (1950). The collapse of the ordered structure of the nucleic acid-free, repolymerized protein on drying (Franklin, 1955*b*) shows that the nucleic acid in some way stabilizes the helical arrangement of protein sub-units. Both these observations accord well with the single-chain, flat-helix hypothesis described above, though neither can be considered to add much additional weight to the hypothesis.

Discussion

It is clearly desirable, not merely to summarize the results which have so far emerged from X-ray diffraction studies of TMV, but, since the research is far from its final stage, to differentiate between hypothetical interpretation and well-founded conclusions.

It is well established that there is a helical arrangement of $3n + 1$ sub-units on 3 turns of the helix. Measurements on mercury-substituted TMV provide strong evidence that n is 16, giving 49 sub-units on 3 turns. Further confirmation of this is found in the form (to be described elsewhere) of the contribution of the RNA to the X-ray diagram of TMV. Since the value of n is of fundamental importance in relating X-ray to chemical data, it is intended to seek yet further confirmation by making measurements on other heavy-atom derivatives of TMV. There is, however, very strong indication that the value, 16, for n is correct; it certainly cannot be in error by more than one unit.

This leads us to the conclusion that, either the particle

molecular weight of TMV is about 40×10^6, or not all the chemical sub-units are included in the helical arrangement suggested.

We wish to emphasize that, while the results described above suggest strongly that the sub-units detected by X-rays are to be identified with the chemical sub-units, they do not require that all the sub-units be rigorously identical. Just as chemical studies indicate a strong chemical similarity of all sub-units, so X-ray measurements indicate a strong general structural similarity. The possibility of minor differences among the sub-units cannot, however, be eliminated. Indeed, there is some indirect evidence that there may be a small number of different kinds of sub-units.

When TMV is broken down by mild treatment with alkali or detergent, a homogeneous end-product of molecular weight about 100,000 (Schramm, 1947; Schramm and Zillig, 1955) is obtained. This probably consists of a group of 6 sub-units. If the protein is further degraded, to a molecular weight of the order of that of a single sub-unit, it can no longer be re-aggregated to form TMV-like rods. If this stable group of, probably, 6 sub-units exists in the same configuration as that in which it will ultimately be incorporated into the TMV particle, the situation of the sub-units at the ends of the group must differ from that of those in the middle. This suggests (but by no means proves) that there may be a small structural difference between different sub-units in the group.

Concerning the external shape of the TMV particle, we regard the existence of some form of helical groove as certain. The form of the groove is such as to permit a substantial degree of interlocking between neighbouring parallel particles (Franklin and Klug, 1956) and the outer radius of the particle is appreciably greater than 75 Å, the mean radius. Beyond this nothing is known of the exact shape of the groove, and the schematic representation in Fig. 2 should not be taken literally.

It is too early to speculate profitably about what is, perhaps, the most fundamental question of all in the problem

of the internal structure of TMV, namely the structural relationship between the protein and RNA in the virus. It is, however, certain that the phosphate-sugar back-bone chain of the RNA lies at a radial distance of about 40 Å from the particle axis The RNA is thus deeply embedded in the virus protein. It is not yet possible to say definitely whether there is a single RNA molecule following the line of the primary protein helix throughout the length of the particle, or whether there are a number of RNA molecules following helical paths which are inclined at a smaller angle to the particle axis.

However, there is a good chance that these questions may be answered in the near future The preparation by biochemists of new heavy-atom derivatives of TMV, and of substances related to it, together with further improvements in the techniques of obtaining and interpreting X-ray diagrams, should eventually lead to the determination of the structural configuration of both the nucleic acid and the protein parts of the virus.

Acknowledgements

We are greatly indebted to the biochemists who have provided us with the many different virus preparations used in this work, and

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DISCUSSION

Williams: I have two comments to make. I believe that these results with respect to the grooved, peripheral contours would explain one of

sort of direct confirmation of your conclusion

virus rods come together side-by-side there should be a fairly deep channel, but there is not—the channel is seen to be rather shallow.

...and not like re-polymerized protein with RNA added at random.

Bawden: Like Williams we have often noticed, in electron micrographs, that separated particles of TMV appeared to be wider than are particles when orientated and packed tightly, suggesting that they can fit into one another

to draw pictures for us.

Watson. Dornberger-Schiff published a paper in 1940 in which she said that TMV must be grooved, pointing out the observed diameter of single particles was always greater than those calculated from groups of particles lying side by side. She postulated a helical structure to explain the grooving. However, her precise model was very wrong. She made the pitch of the helix 68 Å and the diameter 300 Å.

Crick: The paradox is, isn't it, that the little core that you see is so stiff?

Bawden: Is there any necessary conflict? Dr. Franklin is working with intact virus particles, whereas to see this central core with the electron microscope the particles have first to be degraded with detergent or something.

Williams: That is all right, but the overall diameter in the electron microscope seems to be too small.

Franklin: It is not sure that there is more than one RNA strand.

Watson: The problem is whether there is one strand or 15 strands. You might think that if there were 15 strands you would have seen a fraying in electron microscope pictures.

Bawden: But when the virus is really broken down, surely very small fibres do occur. I thought we were shown a picture of some this morning? Isn't the central core possibly an artifact, produced in partially disrupted particles?

Franklin. But if it is a single chain going around in a rather large coil, it is rather hard to see how it would fit in with this apparently stiff core.

Williams: I think that the hazards of surface tension artifacts are great with respect to the electron microscopy, the strands, of course, could pull together into a cord upon drying.

Dulbecco: An electron microscope photograph of the protein without nucleic acid, viewed end-on, showed a hole. What about the particle which still has nucleic acid present, would it show a hole also? Have any observations been made? Because according to the diagram which has been shown, it should not make any difference whether the RNA is in or not, for the size of the hole.

Williams. I don't believe that a 20 Å unit hole would ever show up.

Franklin: 20 Å is a radius, not a diameter.

Dulbecco. In this case, we have to admit that when the RNA goes out, a larger hole remains, which would suggest that some protein goes out with the RNA.

Crick: I think that is unlikely. Dr. Franklin's data show that when you reconstitute the protein alone it is very similar to the complete virus. I think you might see the central hole in the intact virus if you sliced across.

Pirie. About how much of the nucleic acid would you have to say is in your helix? Half of it or what?

Franklin: Within experimental error, all of it. In fact, the integrated value of the RNA peak came out to be about 12 per cent, which is just what one would expect because the density is measured with respect to water, and the density of nucleic acid with respect to water is about twice that of protein.

On this question of the core, from the small amount of data we have

which produces it. In this way one gets a satisfactory, consistent picture This is all done from non-equatorial measurements With the earlier suggestions involving a smaller number of structural sub-units, each of which contained two chemical sub-units, one reached the conclusion that the whole of the diffraction diagram of the equator was due to protein (or nucleic acid) lying at a very small radii, and was therefore forced to conclude that the outer particle of the virus particle was disordered,

MATERIAL IN VIRUS PREPARATIONS NOT NECESSARY FOR THE MANIFESTATION OF CHARACTERISTIC VIRUS PROPERTIES

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So far as is known, viruses only multiply in cells that are themselves metabolizing; an extract containing virus is therefore always likely to contain irrelevant products of the host's metabolism as well. Some of these will be normal components of the uninfected host, some will be by-products of virus multiplication, and some will be products of the host's reaction to infection. The only ones that concern us here are those likely to get carried into the virus preparation, and this can either happen because the properties of the material, for example its particle size or behaviour with precipitants, are similar to those of the virus, or else because the material is attached to the virus.

Normal host components that are simply mixed with the virus need not detain us long. Their properties can be discovered by experiments on the uninfected host and their removal, though it may present considerable technical difficulties, should in theory always be possible. The only point of theoretical interest is the difficulty of deciding what should be used as the normal control tissue. Many virus infections upset the host's metabolism so much that a tissue reduced to a cachectic state by some non-infective agent or by old age might well be a more relevant control than undamaged tissue of the same age (cf. Pirie, 1950*a*).

made preparation. At the one extreme is scopoletin (Best,

1944), a pyrone which is present in much greater concentration in tobacco plants after they are infected with tomato spotted wilt virus; at the other extreme are non-infective proteins that resemble the viruses antigenically. These have been found in extracts from many virus-infected tissues and those accompanying influenza virus (von Magnus, 1953), the Brown-Pearce tumour (Kidd, 1946), turnip yellow mosaic virus (Markham and Smith, 1949) and tobacco mosaic virus (TMV) (Bawden and Pirie, 1945, 1956, Takahashi and Ishii, 1952), have been studied in detail. Their particles are characteristically either smaller or less dense than those of the virus, and it is on these differences that separation depends.

It is now generally recognized that virus infection is a complex metabolic disturbance in which non-infective companion proteins are made as well as the virus. There is no reason to assume that all the non-infective companions have physical properties very unlike those of their viruses, and some may be so similar as to make separating difficult, for it is already known (Bawden and Pirie, 1938) that infective virus preparations can be made non-infective without gross changes in their physical properties. There is statistical evidence that one infective particle of some bacterial viruses is sufficient to initiate an infection; but there is no satisfactory evidence that any virus preparation has been made, every particle of which will generally initiate infection. With plant viruses the evidence is incomplete, many thousands of particles from even the best preparations are necessary Sites on a leaf vary in their infectibility (Kleczkowski, 1950); this suggests that much of any inoculum is bound ineffectively. Much of it is probably wasted. We have therefore no means of measuring the proportion of infective particles in a preparation. Furthermore, there is no reason to assume that the same particles are infective at all sites or on all hosts.

If only a small proportion of the particles in a virus preparation is infective, the fact that most of the particles have a certain shape or size is of doubtful significance. And with pleomorphic viruses, such as TMV, there is no basis for

dogmatism about the range of particle sizes with which infectivity can be associated. There is even less reason for confidence that the properties of the infective particles are the same when they are produced in different hosts. This is often asserted in general articles about viruses, but the evidence is meagre and there is some evidence to the contrary. Thus TMV particles in leaf exudates from different hosts have different average lengths (Johnson, 1951). Bawden (1956) has described a strain of TMV that infects both beans and tobacco plants systemically. Preparations from the two hosts show many significant differences in their properties but revert to the properties characteristic of the other host on being returned to it. Several of the possible interpretations of this phenomenon are relevant to the present discussion. The *infective agent may be only a small proportion of each preparation and the two hosts may make different companions*; part of each particle in either type of preparation may be a host component acquired either *in vivo* or during the separation; infectivity may be a property spread over more than one type of particle in each host and the ratios of the different types may vary with the host, the protein-synthesizing mechanism of the host may have its own plan for the types of protein synthesized (as is suggested by the common appearance of species specific antigenic determinants) and this may be overcome incompletely by the intruding virus. The last possibility is in many ways the most probable. It depends on the assumption that viruses multiply by diverting the normal machinery of the host (cf Bawden and Pirie, 1953) and it extends the scope of discussion greatly, for it also depends on the assumption that part of a virus particle may be specifically inessential and only have to meet rather broad specifications. This is a theme that has already been discussed (Pirie, 1950*b*).

The end products of extensive virus purification often differ significantly from the material that existed *in vivo*. We should not therefore accept the common assumption that anything that can be removed from a virus preparation without loss of infectivity is an impurity. Criteria of such

Procrustean simplicity, if generally applied, would have far-reaching consequences, for there are many structures attached to us, and to which we are attached, that could be dispensed with. The biologically important unit is the one that normally exists in the cell or is effective in conveying infection from cell to cell, even although the simplest form of the virus has great chemical and physical interest. This distinction can be clearly made with tobacco mosaic virus because during "purification" there are changes that can be recognized by the character of the serum precipitate, by precipitation with salts and by measurement of various physical properties.

This limiting state of the virus has been called TMV(L) (Pirie, 1956*a*, *b*). It is the state in which material is generally handled in chemical and physical laboratories, but it would be as well if a description of the properties observed were prefaced by fuller details of ancillary properties and of the technique used in the purification, so that this could be more definitely assured. The fact that a certain laboratory has at one time produced a virus preparation with certain properties gives us no reason to suppose that other preparations of what is ostensibly the same virus will always, or even ever, have the same properties Fine chemical manufacturers find it necessary to state the relevant properties of each batch of the low molecular weight substances with which they deal· *a fortiori* such a statement is necessary with very large proteins.

From among the various types of TMV preparation that can be made it is not possible at present to single out one as the ideal from which all the others are derived by deletion, accretion, aggregation or dissociation. The only reason for *starting the discussion with TMV(L) is that it is the largest* and most stable form of the virus and has fewest components. Its only recognized components are protein, nucleic acid, and the cations needed to keep the preparation neutral. There is no evidence whether the fragments that can be made from it after various types of fission can be fractionated into more than one type of protein and nucleic acid. Some or all of the terminal threonine may have been removed from it, depending

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on the techniques used in the preparation. Apart from this labile threonine, none of the N should become unsedimentable on the ultracentrifuge or unprecipitable with trichloroacetic acid when the preparation is incubated with proteolytic enzymes, and none of the P when it is incubated with a ribonuclease. The preparations normally handled, however, contain material that is attacked by these enzymes (Pirie, 1956*a*). This material can be avoided or removed in various ways. The simplest is to let leaf sap age for a few days at room temperature. From some saps it can be removed nearly completely by freezing, but the treatments most likely to be effective with all saps are heating to 50–60° or the addition of phosphate. These unstable components are largely removed during fractionation by isoelectric precipitation or salting out, but preparations made quickly by ultracentrifugation retain them and various other substances, such as ribonuclease (Pirie, 1956*a*) and some coloured substances (Ginoza, Atkinson and Wildman, 1954). All these can be separated from centrifugally purified virus by incubating it with citrate, phosphate and a few other agents, and ultracentrifuging again. All the methods of separation that have been used alter the serological behaviour and physical properties of TMV; these two processes may not necessarily be connected, but the connection has been invariable so far.

There are four different ways in which these removable components may be associated with the virus. (1) They may be normal but not essential, at any rate when the virus is transmitted mechanically. (2) They may be relics of the virus synthesizing system that remain attached to some virus particles to an extent that depends on the circumstances of the multiplication and extraction. It is only if there is variability in the attachment that this condition differs from the first. (3) The combination may take place after virus synthesis; either normally in the infected cell or as an artifact during the process of virus purification when the virus is exposed to cell components with which, *in vivo*, it was not in contact. (4) There may be no union with the virus, but other materials with

similar properties may simply accompany it. This mechanical [illegible]

more fully. The necessity for a component in a virus depends on the environment in which it is tested Thus Wollman and Stent (1952) find that fresh T4 virus is able to infect *Bacterium coli* in simpler media than virus that has matured. The apparently inactivated, mature virus is, however, able to infect *coli* if tryptophan is added. The nature of the change is not known but it is clearly only lethal under certain conditions of testing and, if the change should turn out to be the loss of a component, that component is inessential so long as tests are made in the presence of tryptophan. Other removals might well be possible if we used a sufficient group of co-factors during testing. Similarly, TMV can be modified by such treatments as acetylation or removal of threonine (Harris and Knight, 1952) without loss of infectivity. It is possible that only those particles that were not infective in the first place are undergoing change in these treatments, it is more probable that the modified particle is restored to its original state when it is introduced into a susceptible cell. The photoreactivation (Dulbecco, 1950; Bawden and Kleczkowski, 1953) of some bacterial and plant viruses probably depends on a similar mechanism. Here virus preparations that have been exposed to ultraviolet light for so long that they give very few lesions when inoculated to test plants kept in the dark, give more than ten times as many if the test is made in the light. With this system there is no reactivation when the virus alone is illuminated. If the origin of the preparation were not known, and if testing in the dark were normal, the preparations might have been said to consist mainly of a non-infective virus companions. Photoreactivation has so far only been found after the damage done by ultraviolet light, and even then not for all viruses. Its importance for this discussion is that differences in the conditions of testing control the decision whether certain particles are virus particles or not.

This dependence of infectivity on the metabolic or nutritional state of the host leads to the suggestion that a component that is plentiful in the host may become dispensible in the virus. To take an extreme case: if the interpretation that Fraenkel-Conrat and Williams (1955) put on their experiments with mixtures of protein and nucleic acid separated from TMV is correct, and if infectivity can be restored by their union *in vitro*, either component might become an infective agent if the metabolism of the host produced, or were stimulated to produce, an abundance of normal protein or nucleic acid. The position is in many ways analogous to that with mutant strains of micro-organisms that have lost the ability to synthesize a certain amino acid and so are "dead" unless that amino acid is supplied in the medium.

The idea that parts of the virus synthesizing system may remain attached to the virus offers a ready explanation for the enzymes sometimes found as inessential components of viruses. It would also explain the host component found in influenza virus (Knight, 1946); this appears to be part of the virus particle (Smith, Belyavin and Sheffield, 1955), and it is relevant to this discussion because different materials from different hosts can fill the same rôle.

Associations taking place during the extraction and purification of the virus are very common (Pirie, 1946, 1949, 1953). They are generally recognized when extracts made in different ways, or from hosts in different metabolic states, yield viruses with different properties; the position is made clearer when it is also possible to make the suspected complex artificially. Thus Carr (1953) found variable amounts of haemin in preparations of fowl tumour viruses and showed that he could increase the amount in the virus by adding haemin at an early stage in the purification; Hoagland, Ward, Smadel and Rivers (1942) increased the amount of catalase in vaccinia virus preparations in a similar way. Ginoza, Atkinson and Wildman (1954) could restore the coloured materials to TMV, from which they had been removed, by adding "purified" virus to sap and isolating it over again.

There is no reason to think that any of the enzymes that have been reported in TMV preparations (cf. Pirie, 1956*b*) are genuine constituents of the virus, but it would be premature to accept the common generalization—viruses do not contain enzymes. This is partly because we may not be looking for the correct type of enzyme, and partly because there is good evidence that influenza virus (Henle, 1953) and the virus of fowl erythromyeloblastosis (Beard, Sharp and Eckert, 1955) contain a polysaccharase and adenosine triphosphatase respectively. The associations formed as a consequence of the virus meeting substances with which it was not in contact *in vivo* are often apparently not fortuitous but shed light on the mechanisms of normal invasion or multiplication. Thus Davenport and Horsfall (1948) suggest that the lung component, with which the pneumonia virus of mice combines when the virus is prepared by grinding up whole lungs, plays a part *in vivo* in controlling the nature and spread of the infection. There is similar but transient combination between influenza virus and a component of egg allantoic membrane (cf. Henle, 1953), but it is destroyed by the virus and so does not appear in the final preparation. Tumour viruses give many examples of capriciously non-infective extracts and of extracts containing virus with apparently modified properties. These variations in behaviour are probably often due to combination between the virus and anti-viral antibody, either circulating or anchored, made by the host (Carr, 1944), but complex formation with normal host components and the production of virus companions probably also play a part. The fact that this literature is extensive, confusing, and not always consistent, should not lead us to neglect the phenomena that are there.

Detailed chemical and physical work has so far been confined to the very stable minority of viruses and they have often been studied after considerable modification. This is a useful beginning, but to give the study generality and make it biologically useful, these academic studies need to be extended to less stable viruses and to viruses in the state in which they

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DISCUSSION

[illegible]

Pirie Is that published?

[illegible]

Andrewes. This was done by Gye and Purdy, using both the Rous and the Fujinami sarcoma, but with the Rous sarcoma you cannot do the test readily using two different hosts The advantage of the Fujinami sarcoma is that you can make these tests using two different hosts and that helps to unscramble this problem

Dulbecco I don't think so At least in the case of the Rous sarcoma, [illegible]

the rabbit Now, *in vitro*, the antivirus serum inactivates virus very rapidly, and irreversibly, whereas the antihost serums will not do anything to the virus

However, if you infect the cells of the chorioallantoic membrane with [illegible]

[illegible]

probably transmit infection from cell to cell *in vivo*. This phase of virus research will only develop when the artificiality of most virus preparations is recognized and when, as a corollary, the extent of the alteration is defined.

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niques

Watson: Do you think that 30 per cent contamination was physically attached to the TMV particles?

Pirie: No, I don't think the preparations that I make, which I begin to think are getting pure, have as much as that. But I think it is possible for people to have that amount in the preparations that they give to the physicist to make X-ray measurements on.

Caspar: I would like to make a comment about this question of purification in relation to the X-ray results. I purified my TMV by two

true TMV is?

Pirie: The minimal one is 0.55 or 0.58 per cent phosphorus.

Watson: But in a recent manuscript, you indicated that some TMV

chicken, you could get infective extracts from it. But he, as various other people had done, found that as time went on the extracts diminished in infectivity, and at the same time, the serum titre went up. It was that that made him correlate the fall in infectivity of tumour extracts with the precipitation or combination of the virus inside the animal

direct action on the virus.

Isaacs: Isn't there a virus which is aggregated by antiserum to the host cell?

virus using serum (a) against a normal component, and (b) against a Forssman antigen.

However, I am inclined to agree that this does not mean that Beard has shown true neutralization, because I think that his method of

Pirie: You can have a preparation from which you would strip

SOME RECENT DEVELOPMENTS IN THE CHEMISTRY OF VIRUS MUTANTS*

C. A. Knight

Virus Laboratory, University of California, Berkeley, California

It has seemed for many years that the different biological properties exhibited by virus strains (also called mutants or variants) must reflect differences in the chemical structures of the viruses themselves. This possibility has been most extensively investigated with strains of tobacco mosaic virus (TMV), which consequently will be made the focal point of discussion here.

The infectivity of TMV has been shown to be associated mainly, if not exclusively, with characteristic rod-like particles whose dimensions are about 15 by 300 mμ (Stanley, 1939; Williams, 1954) and whose molecular weight is about 50×10^6 (Williams, Backus and Steere, 1951). It has been supposed by some that mutation of TMV results in strains whose particles may differ significantly from those of the parent strain in size or shape However, this does not seem to be the case (Bernal and Fankuchen, 1937, 1941, Stanley, 1943; Knight and Oster, 1947). In fact, there is, to the author's knowledge, no convincing evidence that the particles of any virus strains differ from one another in size and shape any more than the particles of any single strain differ from one another, which is slight by present methods of measurement

A pronounced chemical similarity is also characteristic of virus strains. From phosphorus and other analyses, it has been concluded that TMV and 12 of its strains are all composed

* Some of the work cited herein by the author and his collaborators has been aided by grants from the National Institutes of Health, United States Public Health Service

preparations had phosphorus values as low as 0·45. Which do you

pleomorphic.

value is the one which makes the regular structure. Whether this stucture is intimately correlated with the infectivity is a second question.

I should like to ask about your guess. Do I understand that you expect that it will eventually be possible to show that the protein part alone is infective?

Pirie: That was a guess when I wrote the paper. Now it is claimed that RNA without protein can be infective. I do not see why a sufficiently vigorous host might not find other normally non-infective materials, infective.

Crick: This seems to me rather different from the simplified view that

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of about 94 per cent protein and 6 per cent ribonucleic acid (Knight, 1954). Strains of tomato bushy stunt virus were also found to have the same general composition (deFremery and Knight, 1955), although this differs greatly from that of the TMV strains

Progressing from the more general to the detailed composition of virus strains, it was found that strains of TMV often differ in the proportions, and sometimes kind, of amino acids in their protein components (Knight, 1954). However, it is important to note that differences in protein composition did not prove to be an invariable rule for strains. For example, it has not yet been possible to distinguish in any way between the compositions of TMV protein and the protein of the masked strain (M). The proteins of some other pairs of strains seem likewise to be identical in composition, and this lack of distinction appears to apply also to some strains of tomato bushy stunt virus (deFremery and Knight, 1955).

In the case of the nucleic acid components, it has been found that the nucleic acids of 16 distinctive strains of TMV have the same proportions of purine and pyrimidine bases (Markham and Smith, 1950; Knight, 1954; Cooper and Loring, 1954). Similarly, no differences have been found in the compositions of the nucleic acids of 8 strains of tomato bushy stunt virus (deFremery and Knight, 1955), 2 strains of cucumber virus (Knight, 1954), and the T even-numbered bacteriophages (Wyatt, 1953). The nucleic acids of 7 strains of potato virus X were also found to be very similar in composition (Markham, 1953).

To summarize the compositional studies, it can be concluded that mutation of a virus is sometimes, but not always, accompanied by detectable changes in the composition of the protein. By contrast, the nucleic acids of virus strains seem [illegible]

[illegible] has been made between [illegible]ogical properties of TMV [illegible] in protein composition, where they exist, are responsible for observed differences in

the immunochemical properties, and are probably also involved in certain host-virus relationships as evidenced by different rates of movement of strains in a given host and by somewhat different host ranges. In the case of bacterial viruses, host specificity and other properties have been clearly linked to the protein (Herriott, 1951) However, the previously mentioned fact that some virus strains seem not to differ at all in composition, even though their biological properties are quite distinct, suggests that there may be vital differences between strains in the structural features of their protein or nucleic acid components

As a beginning of the search for possible structural differences in the nucleic acids of virus strains, the nucleic acids of

acetic acid (TCA) (Reddi and Knight, 1956) About 14 per cent of each nucleic acid was found in the TCA-insoluble fraction, and analyses showed that the components of these fractions differed substantially in composition from the intact nucleic acids, that they were rich in purine and poor in pyrimidine, and that they averaged about 6 nucleotides in length. Within the limits of the method of analysis used, the TCA-insoluble fractions of all 5 strains appeared to have the same composition. This finding emphasizes in a new way the similarity in composition, and possibly also in structure, of the strain nucleic acids. Clearly, much more work must be done in order to establish whether or not there are significant structural differences in the nucleic acids of strains of TMV; however, even at this stage it is apparent that the strain nucleic acids do not possess grossly different sequences of nucleotides

New information bearing on the structure of TMV, and of its protein in particular, was obtained by treatment of the virus with the enzyme, carboxypeptidase (Harris and Knight, 1952, 1955; Schramm, Braunitzer, and Schneider, 1954). This enzyme caused the specific release of about 2900 threonine

residues per mole of virus. The threonine released is assumed to represent the carboxyl-terminal (C-terminal) residues of a corresponding number of polypeptide chains in the virus molecule. Confirmation of the C-terminal threonine has been made by a strictly chemical method (Braunitzer, 1954; Niu and Fraenkel-Conrat, 1955*a*). From the number of C-terminal threonine residues and on the basis of a molecular weight of 50×10^6, it can be calculated that the virus is made up of about 2900 repeating sub-units whose molecular weight is about 17,000.

Turning to strains of TMV, treatment with carboxypeptidase was found to release only threonine, and this in equivalent amounts, from 13 strains of the virus (Knight, 1955). Unrelated plant viruses similarly treated gave results which were qualitatively and quantitatively distinct from those found for TMV and its strains. Hence, this provides a basis for predicting that strains of a virus will probably possess the same C-terminal amino acid. Of greater significance is the indication that strains of TMV are composed of similar, if not identical, numbers of chemical sub-units.

There appears to be no free amino-terminal (N-terminal) end of the TMV peptide chains, either in the intact virus or in the isolated protein sub-units (Fraenkel-Conrat and Singer, 1954). Hence, it has been concluded that the N-terminal ends of the TMV peptide chains are bound in a ring structure; this ring is hydrolytically split, together with lesser amounts of other peptide bonds, upon heating with trichloroacetic acid to give proline as the main N-terminal acid (Schramm and Braunitzer, 1953; Schramm *et al.*, 1954). The N-terminal amino acid of strains of TMV has not yet been investigated, but from the great similarity of the strains in other respects, it is assumed that they will also be found alike with regard to this structural feature.

Further details of the chemical structure of TMV and its strains are now under investigation. Schramm and Braunitzer (1955) have described the splitting of TMV protein by trypsin to yield 11 peptides including a crystalline one

containing 21 amino acids.* Except for the crystalline one, the peptides were not isolated, but were assumed present from the results of end-group analyses. We have also investigated the tryptic digests of TMV protein, using as starting material the sub-unit protein obtained from TMV by treatment with detergent (Sreenivasaya and Pirie, 1938, Fraenkel-Conrat and Singer, 1954).

The peptides resulting from tryptic digestion of TMV protein have been separated on filter paper by electrophoresis at pH 7 in one dimension followed by chromatography in butanol-acetic acid-water in the second dimension. A typical chromatogram, after spraying with ninhydrin and marking, is illustrated diagrammatically in Figure 1. About 14 spots are consistently observed on such chromatograms and these may include the 13 peptides to be expected on the basis of the specificity of trypsin, which is known to split the peptide bonds involving the carboxyl groups of the arginine and lysine residues Spraying of similar chromatograms with Saka-

to yield ornithine (from arginine), lysine, and threonine as the C-terminal amino acids. Traces of phenylalanine and leucine were also found, which together with two or three fainter spots on the ninhydrin-treated chromatograms indicate a small amount of chymotryptic digestion. It is very difficult to eliminate all of the chymotrypsin from trypsin preparations, but even with the slight contamination, it is clear that tryptic digestion of TMV protein is both quite complete and highly specific.

The tryptic peptides described above are being isolated by various procedures and subjected to analyses for end-groups,

* Schramm and Anderer (1955) calculated that there are

residues per mole of virus. The threonine released is assumed to represent the carboxyl-terminal (C-terminal) residues of a corresponding number of polypeptide chains in the virus molecule. Confirmation of the C-terminal threonine has been made by a strictly chemical method (Braunitzer, 1954; Niu and Fraenkel-Conrat, 1955*a*). From the number of C-terminal threonine residues and on the basis of a molecular weight of 50×10^6, it can be calculated that the virus is made up of about 2900 repeating sub-units whose molecular weight is about 17,000.

Turning to strains of TMV, treatment with carboxypeptidase was found to release only threonine, and this in equivalent amounts, from 13 strains of the virus (Knight, 1955). Unrelated plant viruses similarly treated gave results which were qualitatively and quantitatively distinct from those found for TMV and its strains. Hence, this provides a basis for predicting that strains of a virus will probably possess the same C-terminal amino acid. Of greater significance is the indication that strains of TMV are composed of similar, if not identical, numbers of chemical sub-units.

There appears to be no free amino-terminal (N-terminal) end of the TMV peptide chains, either in the intact virus or in the isolated protein sub-units (Fraenkel-Conrat and Singer, 1954). Hence, it has been concluded that the N-terminal ends of the TMV peptide chains are bound in a ring structure; this ring is hydrolytically split, together with lesser amounts of other peptide bonds, upon heating with trichloroacetic acid to give proline as the main N-terminal acid (Schramm and Braunitzer, 1953; Schramm *et al*, 1954). The N-terminal amino acid of strains of TMV has not yet been investigated, but from the great similarity of the strains in other respects, it is assumed that they will also be found alike with regard to this structural feature.

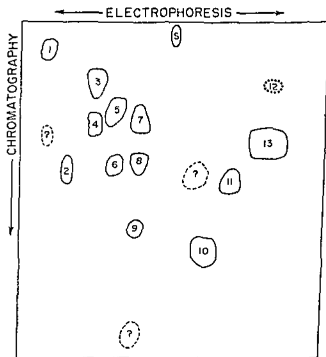
Further details of the chemical structure of TMV and its strains are now under investigation. Schramm and Braunitzer (1955) have described the splitting of TMV protein by trypsin to yield 11 peptides including a crystalline one

with the most divergent amino acid compositions, namely TMV and HR,* it was found, as expected, that the peptide patterns were grossly different, having only a few similarities. Next, comparing TMV with the strain, YA, which appears to differ from TMV only in having 1 more arginine and 1 (or 2) less isoleucines per protein sub-unit, it was found that very similar peptide patterns were obtained. Nine of the peptide 3 or 4 differ. Finally, se amino acid content the same distribution of peptides was found In fact, a mixture of tryptic digests of the 2 strains yielded the same pattern of peptides as given by TMV alone (see Fig. 1)

Studies are also being made of the products resulting from chymotryptic digestion of TMV and some of its strains. Chymotrypsin, it will be recalled, splits peptide bonds next to tyrosine and phenylalanine, and to some extent leucine (Sanger and Tuppy, 1951). After chymotryptic digestion of TMV protein, 20 or more peptides can be detected by ion exchange chromatography (Niu, unpublished data) or by paper electrophoresis and chromatography In either case, a method to separate cleanly all of the peptides has not yet been worked out and hence, chymotryptic digests of TMV strains have not been surveyed in the manner of the tryptic digests as illustrated in Figure 1. On the other hand, dinitrophenylation of the mixture of peptides released by chymotryptic hydrolysis of TMV protein and certain of the strain proteins permitted the isolation by two-dimensional paper chromatography of several peptides (Niu and Fraenkel-Conrat, 1955b). After identifying a certain peptide as a hexapeptide derived from the C-terminal end of TMV peptide chains, attention was directed to similar products obtained from the strains M, YA, and HR The hexapeptides from TMV, M, and YA proved to be identical, having the sequence

* The protein of the HR strain was found to differ from that of TMV in the proportions of 13 of the 16 amino acids which they contain in common, and in addition was found to contain histidine and methionine, amino acids entirely lacking in TMV (Knight, 1947)

amino acid content, and sequences of the amino acids. In the meantime, however, significant comparisons have been



made of the two-dimensional patterns obtained by paper electrophoresis and chromatography of the tryptic digests of several strains of TMV. Starting first with a pair of strains

ever, insulin has only 2 chains whereas TMV has about 3,000, and it may be that the repetition of a small change several thousand times would affect the biological properties of a macromolecule. But the tryptic digests of TMV and M seem to yield exactly the same peptides, which presents a case just the opposite of that for the insulins. The insulins represent chemically different molecules with apparently the same biological activity, whereas the virus proteins seem to have identical chemical structures but very different biological properties. Quite possibly the structural studies on the virus proteins have not progressed far enough to reveal subtle differences; on the other hand, the key may lie in the nucleic acids, since TMV and its strains are nucleoproteins A decisive rôle has in fact been claimed for the nucleic acid by Fraenkel-Conrat (1956) as a result of experiments on the reconstitution of virus from the protein of TMV and the nucleic acid of HR. The symptoms produced upon infection of tobacco with such a combination proved to be characteristic of HR. Moreover, it was suggested that the nucleic acid alone is infectious and causes a disease characteristic of the whole virus from which the nucleic acid was derived. Gierer and Schramm (1956*a*, *b*) have also claimed that TMV nucleic acid alone is infectious.

However, from the point of view of virus replication, it is difficult to picture how two biologically distinct strains of virus, like TMV and M, could have either identical protein or identical nucleic acid components It seems more reasonable to assume that a difference in one component would be reflected in a difference in the other. It is apparent that further structural studies must be made on both protein and nucleic acid components before a complete chemical picture of virus mutation can be drawn.

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threonyl - seryl - glycyl - prolyl - alanyl - threonine, while the sequence of the HR hexapeptide is threonyl (threonyl, alanyl)-prolyl-alanyl-threonine. Thus, the strain which differs most from TMV in amino acid content, HR, has some differences in amino acid sequence as near as the fourth amino acid in the 145-residue chain, whereas the sequences of strains similar to TMV in composition continue the same for at least several residues farther. It has occasionally been suggested that two strains such as TMV and M might have the same amino acid content, but quite different sequential arrangements of the amino acid residues. Although the present data are very fragmentary, it is already clear that there is not a complete reshuffling of sequences of amino acids in the proteins of TMV and M, nor in YA either.

Few conclusions can be drawn as yet regarding the chemical structure of the proteins of TMV strains. However, as mentioned earlier, it seems from the quantitative determination of C-terminal groups, that the various strains consist of essentially the same number of chemical sub-units The next question (assuming uniformity of the sub-units) is how similar or different are the sub-units of different strains? No matter how closely or distantly related in biological effects the strains may be, their protein sub-units share certain characteristic features. Thus, the sub-units for all strains terminate in threonine, and it has been shown for four strains that this identity of structure extends back 3 amino acid residues into the approximately 145-residue chain, and for 3 of the 4, the same sequence is found for the first 6 C-terminal residues and may extend appreciably further.

One may wonder then to what extent the biological activity of a virus is affected by a few changes in amino acid sequence along a rather extensive peptide chain. If one reasons from the findings on insulin, it might be concluded that small changes have no effect, for it was shown that insulins from 3 species which differ structurally only in the sequence of a 3-residue segment of one of the peptide chains possess identical biological activities (Brown, Sanger and Kitai, 1955). How-

conditions in which infected plants are grown. In the same way, many variants can be separated, by using local single lesions in *Nicotiana* [illegible]

point.

Burnet. Could we have some information as to what serological differences, if any, there are between these strains?

Knight: A good many of the strains can be distinguished serologically and were, in fact, many years ago by Bawden and Pirie. Some of them are distinguished with difficulty, for example the J 14 D 1 and the [illegible] with difficulty, if at all, from TMV [illegible]

Bawden. Mainly because they are serologically related. But they also protect plants against one another, are morphologically indistinguishable, and they have the same general chemical and physical properties

[illegible]

Knight: Yes.

Pirie: With our strains you don't

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DISCUSSION

[illegible] Among the strains in which you find differences, do you con-

[illegible]

tomato plant. It is easy to isolate variants from it that cause different kinds of symptoms, and the symptoms they cause also depend on the

Knight: In Berkeley, I would say that we have less tendency for the masked strain to revert to ordinary TMV

Hawden: Possibly because your glasshouse temperatures are higher than ours.

Knight : Yes.

they cause much more severe symptoms

Hawden: You would perhaps agree that the common strain is the common one because it reaches the highest concentration and is the most infective.

Knight. Yes.

Watson: Were the masked strain and the TMV grown in the same host?

Knight. Yes, in what we call Turkish tobacco.

Isaacs. Do you get the same competition if you grow the same virus in two different hosts?

Knight. Yes, although [illegible]

whether we grew the virus in phlox or in tobacco We have also analysed the nucleic acid components derived from virus obtained from phlox and tobacco and find them to be indistinguishable

Williams. Dr. Knight, have you ever done any amino acid analysis of the X-protein?

[illegible]

antisera prepared against TMV, enation mosaic and aucuba mosaic [illegible]

from certain, because the antibody content of a serum depends on the rabbit used, the number of injections it gets and the amount of virus injected. When only a small amount is injected and the serum has a small titre, virus strains often seem to be less closely related serologically than when a lot is injected and sera have large titres, for when much virus is injected antibodies are increasingly produced against quantita- [illegible]

from ordinary [illegible]

Knight: In Berkeley, I would say that we have less tendency for the masked strain to revert to ordinary TMV

Bawden: Possibly because your glasshouse temperatures are higher than ours.

Knight: Yes.

Spooner: Does the masked strain when it is growing in a plant produce any symptoms? Does it produce as much virus?

Knight: The masked strain when grown in Turkish tobacco produces no symptoms and hence the term "masked".

they cause much more severe symptoms

Bawden You would perhaps agree that the common strain is the common one because it reaches the highest concentration and is the most infective.

Knight. Yes

Williams What is the recent story on that which is neither nucleic acid nor protein in TMV? Do your analyses add up to 100 per cent?

Knight We had it close to 100 per cent on our original amino acid analyses but we allowed ourselves 5 or 10 per cent variation on each

get rid of it The problem I was concerned with was whether it is a genuine component or gets in during the preparation

Markham. Doesn't the DNA just account for about 4 nucleotides per particle? It would be very small

Pirie Yes

GENERAL DISCUSSION

Crick Mr. Bawden, you said that the A-protein was an inhibitor. I should be interested to hear more about that

ultracentrifuged when the preparation has about 10 per cent of the original infectivity, there is a birefringent pellet of nucleoprotein that is infective, but weight for weight, less so than the original virus preparation.

it will not cause lesions because infection is inhibited by the other protein there

Crick · You do not know, in your infectious case, whether you have got any RNA?

Bawden · There is some nucleic acid left in the preparation. Tests for nucleic acid work down to about 5 per cent of the original nucleic acid in the virus preparation; if there is less than this, some people say there is none, some say there is less than 5 per cent.

It may be that the incubation with ammonium sulphate produces

Pirie: The one-sixth would have come down into the bottom layer probably. It would move down the tube but it wouldn't compact into a pellet so that if you simply poured off the supernatant you would say it had not sedimented, if you siphon off the top layer you would say that it had

Watson: But you siphoned off the top layer, is that right?

Pirie. Yes.

Watson: And this contains no activity?

Pirie: If tested crudely enough.

Watson. You add ammonium sulphate and this causes the formation of the small precipitate?

Pirie: Yes, near enough. The results vary according to which precise way you do it—if you add sufficient ammonium sulphate to get only partial precipitation, the ammonium sulphate precipitate on its own is not notably infectious, but if you now ultracentrifuge it, only a part of it sediments and it is that sediment from the ammonium sulphate precipitation which appears to recover quite a [illegible] sulphate in 4 stages to get successive fractions, it is out of the second fraction that the infective material is obtained.

Smith Mr. Bawden, if you take these different fractions at serial time intervals, is there a quantitive increase of the inhibitory substance?

Bawden: Yes The inhibiting protein is produced in proportion to the amount of the virus broken down by exposure to alkali.

Watson You mean you are not just selecting for a certain fraction already there?

Bawden How could we know for certain? But I think it is at least as possible as that we are [illegible]

[illegible]

time taking only the supernatant so as to clear away the intact rods. The second supernatant is then given a third sedimentation and the supernatant fluid poured off. Presumably anything that existed in this second supernatant of a length comparable to what we are talking about, would be thrown down to the bottom of the centrifuge tube The top material from the third sedimentation is poured off carrying with it the protein *and* the inhibitor, and the material at the bottom of the tube, when taken up with water and tested for infectivity, shows none I think this procedure would eliminate any doubt but that protein can be prepared that by itself is non-infectious.

Bawden Yes. I think there is little doubt of that.

Williams. The interesting thing that you report is that apparently the nucleic acid which comes out of TMV when prepared by the high pH treatment is in a state such that, when it and the protein are put back together by treating with ammonium sulphate, followed by centrifugation, infectivity results Can you think of any reason why Schramm failed to get infectivity when he tried this re-assembly many years ago?

Bawden. Yes, I think it was because he tested for infectivity in the presence of protein that inhibits infection. If the protein is wholly aggregated, which can be done by incubating with phosphate or, better still, with trypsin, the sedimented pellet will not be infective when resuspended. It is only when some fractionation, such as your method of incubating with ammonium sulphate, makes a critical part of the preparation sedimentable that an infectious component is detectable.

Salton Do I understand that trypsin does not digest the protein inhibitor?

Bawden Certainly not readily, but I don't know if it is as resistant as intact TMV

v. d Ende I wonder if there is not confusion between two lines of argument Mr Bawden, I think, has explained one possibility very clearly, that there may be a residue of infective particles in the nucleic acid preparation which are removed from an inhibitor by centrifugation. Dr Williams, this morning, explained to us that if he takes the deposit from his preparation which he presumes contains nucleic acid only, he can find no rods in it at all; but he can find rods in fresh virus preparations which have a lower infective titre than his nucleic acid preparations If the infectivity of the nucleic acid depended on the presence of residual rods as suggested by Mr. Bawden there should be enough to be seen by electron photomicrography.

Williams: I was referring this morning primarily, in the quantitative statements that I attempted to make, to assaying the nucleic

acid fraction for the presence of rods Actually, the quantitative examination of such material by electron microscopy is rather sensitive and I think I can point that out by a numerical example. If one starts with a preparation of RNA whose concentration is, say, 1,000 μg./ml , and if one sediments this at high speed to throw down whatever there is in the way of TMV-like particles, this sedimentable material can be taken up in about a tenth the initial volume. Effectively then we can examine whatever has sedimented from nucleic acid at a concentration of 10,000 μg /ml We examine the material that has been thrown down from the initial nucleic acid solution and then taken up in a small volume of water

no less than 10^9 TMV particles/ml, we feel that the electron microscope assay gives us a thousand-fold factor of safety.

Watson In the last group, do you see many particles 500 Å in length?

this question of the virus being intact and concentrate instead on seeing how pure you can get your RNA preparation and still get it infective The real point is what techniques can you use to remove the 1 per cent of the protein? I do not know whether, say, trypsin would remove sufficient of it

Does papain attack TMV protein?

Bawden No; nor has any other enzyme we have tested destroyed TMV

Williams I think the difficulty is that the protein which remains is probably not the fully broken-down unit of molecular weight

migrate at different rates

I should like to ask Dr. Williams if there is a possibility that nucleic acid preparations could be contaminated with significant

numbers of virus rods and that these rods might escape detection in the electron microscope. My thought is, the virus is known to have a tendency to aggregate on the acid side of the isoelectric point, and one dilutes these things for spraying in electron microscopy with water which is on the acid side. Is there any danger that intact virus rods might aggregate under such conditions thus introducing severe sampling problems into the spray drop counting techniques?

Williams Does aggregation take place severely at pH's of the order of 5 5? I thought you had to drop considerably lower than that.

Knight: No, it tends to aggregate as soon as you get below neutrality, and it gets progressively worse as you go down.

Williams. Observations have not been made with material at higher pH, but they should be.

Schäfer: Gierer and Schramm (1956 *Z. Naturf.*, **11b**, 138) have recently done some work on the infectivity of the isolated nucleic acid fraction from TMV They showed that in contrast to the native TMV particle, the infectivity of their nucleic acid fraction is destroyed by RNase; furthermore, the infectivity of the nucleic acid fraction is not neutralized by TMV antiserum, which neutralizes intact TMV. Finally they showed that their infective fraction is sedimented in the ultracentrifuge like DNA rather than like TMV. I think that these are three points in evidence for the activity of the nucleic acid fraction

Williams: The similarities between that report and the work done by Fraenkel-Conrat which I am reporting here, are the differences of sedimentation behaviour between the RNA fraction and

fective, but whether it requires the presence of some protein to

ive for the infec-

le? How does it

was about 2 per

we heard earlier

it is clear that the RNA and protein are pretty tightly fitted together. When you separate the RNA and protein, and then reconstitute the protein back into rods, it is antigenetically unchanged?

not fit the part?

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Sto
differ
new t
might be a slight alteration in the configuration of the original

nucleoprotein rather than the reconstituted protein.

Stoker Yes, but there are two rather different questions here, I think. You might expect a less orderly reconstitution without any nucleic acid, but I also wondered if the nucleic acid from B, for

protein either to protect it or for some mechanism to enable it to enter the cell. But exceptionally it may come across a cell which is damaged in some way, or in a particular state of multiplication, or having something abnormal about it, so that just occasionally the RNA gets put into the right place on its own accord. Would that not fit the part?

can detect the injury is that the nucleic acid of these rods is now

material.

Harington: Yes.

Williams: Hart's experiment is a nice one, because, with respect to the reconstitution, it leaves entirely out of the question the possibility that the nucleic acid fraction used in the reconstitution is contaminated. Hart doesn't use any separated ribonucleic acid fraction, but rather only that RNA which is still attached to the virus.

Sanders: Does this in any way suggest that the function of the protein is to help the RNA into the cell or simply to protect it from damage? After all, the introduction of virus into a plant cell is a pretty rugged process, and I feel that the function of the protein may be simply to protect the RNA until it has been thrust through the cell wall into the interior cytoplasm.

Williams: Is it also possible to speculate that perhaps within the cell the infectious unit, in travelling from one cell to the other in a systemic infection, travels as RNA rather than as the entire TMV rod. This might make the technical translation somewhat more simplified.

Burnet: There is one point which interests me here. Assuming that

microscopy the whole affair is impossible at the present time, since

for obvious reasons. Schachman has been doing this work with ultraviolet optics *for which the dilution of the RNA is of the order for* which one finds the very rapid decay of infectivity with time, and so

the whole thing is uncertain at the moment; one doesn't know the answer.

Harris: Mr Bawden, is the inactivating effect of RNase on the native virus completely reversible?

[illegible]

powerful an inhibitor as RNase.

Harris: At what pH was the trypsin experiment carried out?

Bawden: At pH 7 or 8, values where trypsin is proteolytically active.

Harris: In my own experience the pH at which the native virus is exposed to the action of proteolytic enzymes appears to be rather critical. In the case of TMV it is probably true to say that the [illegible]

[illegible]

lyse; they are also hydrolysed by pepsin and papain.

Harris: In the native state?

Bawden: Yes, potato virus X quite readily, but not by ribonuclease.

Harris: Can you be sure that trypsin is attacking the native virus? Is it not possible that, as in the case of TMV, some kind of base-catalysed denaturation reaction has to occur before the virus becomes a substrate for the enzyme?

Pirie: Potato X is rather more stable in alkali in the absence of trypsin. TMV always goes off from 8.5 onwards, whereas potato X goes up to about 9.5 or so.

can detect the injury is that the nucleic acid of these rods is now partly susceptible to ribonuclease digestion, whereas before treatment with detergent it is not.

Harington But Andrewes was talking about injuring the cell, and making it more accessible to the RNA.

Knight. But the Hart experiments would seem to indicate that the protein has something to do with the infectious quality of the material.

Harington: Yes.

Williams Hart's experiment is a nice one, because, with respect to the reconstitution, it leaves entirely out of the question the possibility that the nucleic acid fraction used in the reconstitution is contaminated. Hart doesn't use any separated ribonucleic acid fraction, but rather only that RNA which is still attached to the virus.

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Burnet. There is one point which interests me here. Assuming that these RNA preparations are infective by virtue of the RNA in them, does this provide any evidence in regard to the length of nucleotide chains necessary to carry the full genetic determinants of the virus?

UNITS ISOLATED AFTER SPLITTING FOWL PLAGUE VIRUS

WERNER SCHÄFER
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IN establishing biochemical reaction chains, the first step is usually to isolate the different constituents, the second to investigate their structure. Finally one has to establish the sequence of the constituents and the mode of their formation. In a similar way we try to elucidate the reactions which lead to the multiplication of an animal virus.

For several years the virus of classical fowl plague (KP) has served as a model for animal viruses in our laboratory. Three different types of particles which appear during the multiplication of KP virus have been isolated in a highly purified form: the infectious elementary particle (Schafer, Munk and Armbruster, 1952), the soluble antigen (s-antigen) (Schäfer and Munk, 1952*a*), and the so-called 'incomplete forms' (Schafer, Zillig and Munk, 1954). The 'filamentous particles' which are also observed (Hotz and Schafer, 1955) have not yet been isolated.

The next step was the investigation of the structure of the isolated particles. We began with the study of the elementary particle (Schafer and Zillig, 1954, Zillig, Schafer and Ullmann, 1955), which is the initial as well as the final product of the multiplication process.

One gets a better understanding of the structure of the virus particle by *splitting the particles and examining* the degradation products This has been done, for example, with tobacco mosaic virus (Schramm, 1947; Gierer and Schramm, 1956). KP elementary particles can be split with ether—the method also used by Hoyle (1952) for the degradation of influenza virus. Two main products have been isolated and studied in detail.

Stoker. Is anything known about the distribution of ribonuclease in the plant cell?

Pirie The leaf cell is rather rich in ribonuclease, and tobacco leaves, anyway, are poor in protease. In fact, it is rather difficult to demonstrate any protease in the tobacco leaf at all; you can do it but it is not easy.

Stoker Is the ribonuclease distributed in any particular place in the cell?

Pirie: There is some concentrated on the microsomes but only a fifth, say, of the total. Most of it appears in the ultracentrifuge supernatant from leaf sap (Holden, M., and Pirie, N. W. (1955). *Biochem. J.*, 60, 39; Pirie, N. W. (1950). *ibid.*, 47, 614).

Williams: With respect to the ribonuclease in the plant cell, I suppose this might well account for the great difference in infectivity between the RNA itself and the intact virus. I presume that the juice that comes out of the leaf upon mechanical inoculation would contain ribonuclease.

It is clear there are two difficulties here: one is that you have to have a method for removing just the end, and this is technically tricky; it might possibly be done by damaging the virus very slightly by reacting with one of the enzymes which attack RNA. The other is the danger that, even when you have done it, a protein of that comosiption might not be replicable—it might not fold up properly for example. My point is that the *ends* of the molecule of RNA are about the only places you can get at to characterize it at all.

UNITS ISOLATED AFTER SPLITTING FOWL PLAGUE VIRUS

WERNER SCHÄFER

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IN establishing biochemical reaction chains, the first step is usually to isolate the different constituents, the second to investigate their structure. Finally one has to establish the sequence of the constituents and the mode of their formation In a similar way we try to elucidate the reactions which lead to the multiplication of an animal virus.

For several years the virus of classical fowl plague (KP) has served as a model for animal viruses in our laboratory Three different types of particles which appear during the multiplication of KP virus have been isolated in a highly purified form: the infectious elementary particle (Schäfer, Munk and Armbruster, 1952), the soluble antigen (s-antigen) (Schäfer and Munk, 1952*a*), and the so-called 'incomplete forms' (Schäfer, Zillig and Munk, 1954) The 'filamentous particles' which are also observed (Hotz and Schäfer, 1955) have not yet been isolated.

The next step was the investigation of the structure of the [illegible]

multiplication process.

One gets a better understanding of the structure of the virus particle by splitting the particles and examining the degradation products. This has been done, for example, with tobacco mosaic virus (Schramm, 1947; Gierer and Schramm, 1956). KP elementary particles can be split with ether—the method also used by Hoyle (1952) for the degradation of influenza virus Two main products have been isolated and studied in detail

In the following I wish to give a survey of the properties of the products obtained in comparison with those of the intact particles. The localization of the degradation products in the elementary particle will be discussed. Finally I want to point out how far the elementary particle and its components correspond to the s-antigen and the 'incomplete forms'.

Methods

Highly purified concentrates of KP virus 'Rostock' obtained from infectious egg fluid were shaken up with ether at 37° C for some hours. The ether must be free from peroxides. The water-phase contained two products, characterized by their different biological activities In accordance with Hoyle (1952), one was named haemagglutinin; the other was called 'gebundenes Antigen' (g.-antigen) in contrast to the s-antigen, which exists as an independent unit in the infected tissue. The haemagglutinin can be separated from the *g.-antigen by adsorption on red cells or by preparative electro*phoresis. After separation the two units were purified and concentrated by fractional centrifugation (Schäfer and Zillig, 1954).

The preparations were examined physicochemically by the Svedberg method, u.v. spectrophotometry, electron microscopy, and partly also by electrophoresis. Chemically we concentrated on determining the type of nucleic acid and the amount. The biological examination included the haemagglutination (H.A) test, the infectivity test, which was done in embryonated eggs, and the testing of the seroimmunological behaviour. For the examination of the immunizing power formol vaccines were prepared and inoculated into chickens in serial dilutions. Fourteen days later, the state of immunity of the animals was tested by infection with $\sim 10^6$ MLD_{50}, and thus we determined the minimal protective 50 per cent dose (MPD_{50}) (Schafer, 1955*b*).

For the serological studies we used five antisera which were obtained from mice and rabbits.

KP mouse serum was obtained from mice infected with a

mouse-adapted KP 'Rostock' virus. Since this virus multiplies in mice, the serum is expected to contain antibodies against all virus-specific units, which show antigenic activity in the course of virus multiplication.

Another serum (FM/1 mouse serum) was obtained from mice, which had survived an infection with influenza FM/1 virus, passaged in mouse lungs (Schäfer, 1955*a*) This serum reacts, as will be demonstrated later, only with certain units of KP.

All the other sera originated from rabbits, immunized with highly purified preparations of either intact elementary particles (KP-rabb.-serum) or with g.-antigen (g.-antigen-rabb.-serum) or with haemagglutinin (H.A.-rabb.-serum). All rabbit sera were absorbed with the 'normal component' of egg fluid in order to eliminate non-specific reactions (Munk and Schäfer, 1951)

The mice sera were used for complement fixation (CF) tests (Schafer, 1951), the rabbit sera for precipitation reactions according to the quantitative technique of Heidelberger (1939).

Properties of the Virus Elementary Particle and its Degradation Products

The infectious unit of KP, i e the virus elementary particle, is a spherical particle 70–80 mμ. in diameter as shown by electron microscopy (Fig. 1) and the Svedberg method. Our latest data show 3–4 per cent RNA Small amounts of DNA which were occasionally found are probably due to impurities The lipid content of the particles is relatively high: about 25–30 per cent [illegible]
to $1 \cdot 4 \pm 0\ 5 \times$ [illegible]
reaction $1 \cdot 58 \pm$ [illegible]
expressed as N, is [illegible] g. In the CF test $44\ 6 \pm 25\ 2 \times 10^{-8}$ g N are needed for a positive result in an assay with KP mouse serum, whereas with FM/1 mouse serum one has to take 18 times the amount of antigen (Table I). The elementary particles are precipitated by

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reaction $1\ 58 \pm 0 \cdot 98 \times$
expressed as N, is 300
$44 \cdot 6 \pm 25 \cdot 2 \times 10^{-8}$ g. N are needed for a positive result in an assay with KP mouse serum, whereas with FM/1 mouse serum one has to take 18 times the amount of antigen (Table I). The elementary particles are precipitated by

Table I

Properties of the Virus-Specific Units

Unit	Form	Diameter ($m\mu$) from a) electron microscopy b) S_{20} and D_{20}	Nucleic Acid %	Infectivity test g N/MLD_{50}	H A test ×10[illegible] g N/H A	Immunization test ×10[illegible] g N/ MPD_{50}	C F test ×10[illegible] g N/pos CF with: KP mouse serum	FM/1 mouse serum	Precipitation with rabbit serum: KP	H.A.	G-Antigen
Elementary particle	sphere	a. 72 ± 7 1* b. 70	RNA 3–4	1 4±0 5 $\times 10^{-15}$	1 53 ±0 98	300 ±109	44 6 ±25 2	770 ±350	+	+	—
Haemagglutinin	sphere	a. 33 7±7 4 b 29 5	—	—	0 07 ±0 02	400	7 9 ±4 9	—	+	+	—
G.-Antigen	sphere (forms aggregates)	a. 10–15	RNA 10–15	—	—	3200	5 1 ±3 1	5 2 ±4 1	—	—	+
S-Antigen	sphere (forms aggregates)	a. 10–15	RNA 6–14	—	—	—	7·0 ±3 0	9 9 ±6 9	—	—	+
'Incomplete forms'	balloon-like	a. 50–550		—	1 2 ±0 60	—	39 ±19	~2000	+		

* = σ

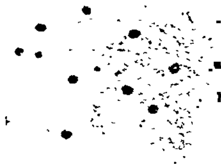


FIG 1 KP elementary particle



FIG 3 Haemagglutinin from elementary particle



FIG 6 G-Antigen from elementary particle

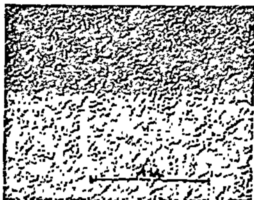


Fig 8 S-Antigen



Fig 10 'Incomplete forms'

anti-KP as well as by anti-H.A. rabbit serum but not by the g.-antigen serum (Fig. 2).

The haemagglutinin from elementary particles has a diameter of about 30 mμ (Fig. 3) Nucleic acid could not be

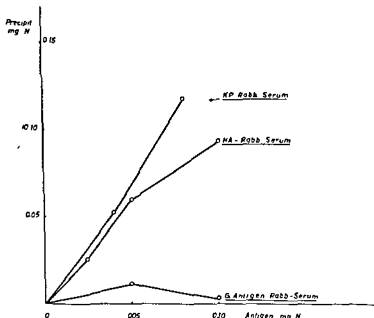


FIG 2 Precipitation of the KP elementary particle

detected (Fig 4). Haemagglutinin alone or in mixture with

specific immunizing activity as the elementary particle, whereas the specific haemagglutinating activity is approximately 20 times larger (Table I) It contains the receptor-destroying enzyme of the virus particle and shows nearly the same serological behaviour as the intact particle. Like the

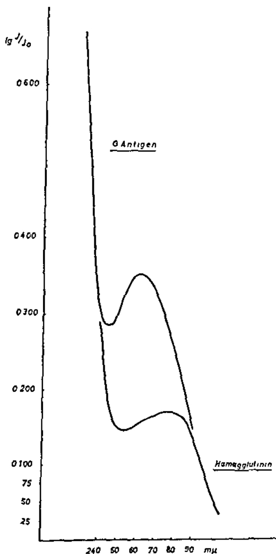


FIG. 4. Ultraviolet spectra of haemagglutinin and g.-antigen.

elementary particle it is precipitated only by KP and H A. rabbit serum (Fig. 5). A positive CF is obtained by KP

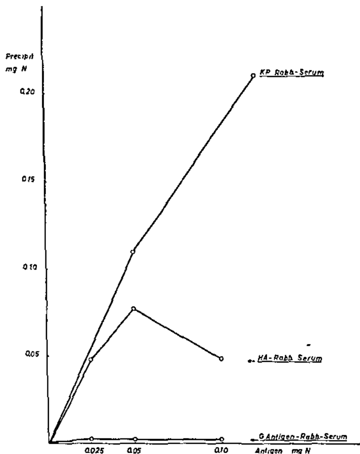


Fig 5 Precipitation of the haemagglutinin

not by FM/1 mouse serum. With the latter serum, as previously mentioned, the elementary particles gave a positive

test only when relatively high amounts of antigen were used.

The g.-antigen is still smaller than the haemagglutinin. According to electron microscopy its diameter is about 10–15 mμ. (Fig. 6); as yet we have not been able to determine the particle size by Svedberg methods because the g.-antigen tends to form chain-like aggregates. It contains about 10–15 per cent RNA and is probably the only nucleic acid-containing component of the infectious unit (Fig. 4). Biologically the g.-antigen can only be demonstrated by serological tests (Table I). With the exception of the CF test with KP mouse serum, its pattern of serological behaviour is different from those of the intact elementary particle and of the haemagglutinin. Thus in the CF test with FM/1 mouse serum even a very small amount ($5 \cdot 2 \pm 4 \cdot 1 \times 10^{-8}$ g. N) of g.-antigen gives a positive reaction. Of the three rabbit sera, only the homologous serum precipitates the g.-antigen (Fig 7) So far it has not been possible to ascertain whether the g.-antigen can give protection to chickens against KP. It seems doubtful whether the protecting capacity discovered is a property of the g -antigen itself, because the relatively high MPD_{50} (3200×10^{-8} g. N) may easily be due to small impurities in the preparations used for the test.

The question can now be raised as to where the degradation products are located in the elementary particle. The haemagglutinin must be part of its surface, because the reactions taking place on the surface of the elementary particle can be reproduced with isolated haemagglutinin. This is the case with the fixation on cell receptors and with the receptor-destroying enzyme activity. The localization of the haemagglutinin can be demonstrated even more clearly seroimmunologically. Thus antihaemagglutinin rabbit serum not only precipitates the haemagglutinin, but also the intact elementary particle. Furthermore, antibodies against haemagglutinin are produced even when intact elementary particles are injected into rabbits. Here the virus does not multiply. Therefore it is likely that only antigen of the virus surface can

be immunologically active. In the elementary particle, the g.-antigen cannot under the same conditions elicit the production of antibodies. Furthermore, the specific antiserum against the g.-antigen does not precipitate intact elementary particles in contrast to antiserum against haemagglutinin. We therefore suggest that the RNA-containing g.-antigen is

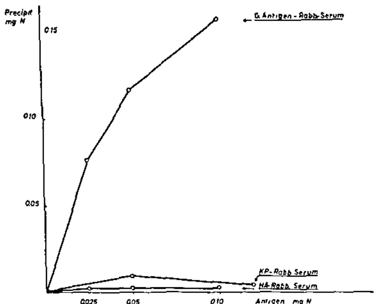


FIG 7 Precipitation of the g.-antigen

located inside the elementary body. The results of the CF test are in agreement with this suggestion. FM/1 mouse serum does not react with haemagglutinin but with g.-antigen. In order to get a positive CF with FM/1 serum and concentrated elementary particles, one needs a relatively high amount of the latter. But upon shaking up the concentrates with ether, their antigenic activity in CF is markedly increased (16–32 fold). This result suggests that the original concentrates

contained a few elementary particles, the g.-antigen of which was accessible to antibodies. The ether extracted the lipids, which act as a binding material, and incidentally liberated the g.-antigen of the other particles.

Apparently the function of the haemagglutinin is to bring the reproductive material of the virus in contact with the host cell. The g.-antigen, however—which is located inside the particle—due to its RNA content must be closely related to the reproductive system. Concerning its structure, it is only this sub-unit of the KP virus which corresponds to the infective particles of smaller viruses like most plant viruses.

It is hoped that a future detailed chemical analysis of the two degradation products will provide further knowledge of the chemical groupings responsible for the different biological functions of the virus. Such investigations might also provide quantitative data on the amounts of g.-antigen and haemagglutinin contained in the elementary particle. According to our present knowledge of the RNA content, the elementary particle contains about 20–40 per cent g.-antigen. This value, however, is valid only under the premise—which still has to be strengthened—that the g.-antigen is the only RNA-containing component of the virus particle.

Comparison of the Virus Elementary Particle and its Degradation Products with other KP-specific Units

As mentioned earlier, two further virus-specific particles could be isolated besides the elementary particle from the infected tissue, namely the s-antigen and the so-called '*incomplete forms*'.

As proved by physicochemical (Figs. 8 and 9), chemical and serological methods (Schäfer, Munk and Mussgay, 1956), the s-antigen has so many properties in common with the g.-antigen from elementary particles (Table I) that doubtless these two units bear a close relationship to each other. The only difference that appeared in vaccination experiments is, as already discussed earlier, not significant. The close relationship between the two particles favours the hypothesis that the

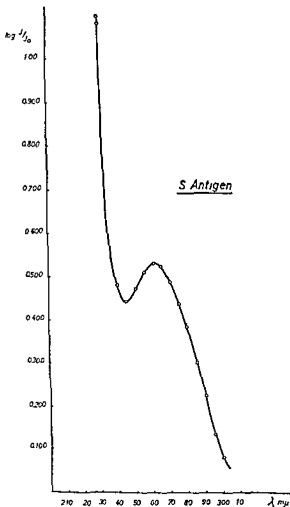


FIG 9. Ultraviolet spectrum of s-antigen

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tion of the different virus-specific units is understood. Labelled virus (Weeker and Schäfer, 1956) will prove useful in reaching this goal.

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[Discussion of this paper was postponed until after the paper by Mr. Ada —Ed]

s-antigen in KP is the RNA-containing unit of the virus, which is released from it during infection and then multiplies in the host cell.

For some time it has been known that the 'incomplete forms' are non-infectious but able to haemagglutinate and that they sediment more slowly in the centrifuge than elementary particles (Schafer and Munk, 1952*b*) Since the same is true for haemagglutinin (Table I), the 'incomplete forms' and the haemagglutinin could be identical. But closer examination, especially by electron microscopy (Figs. 3 and 10), proved both to be different; in contrast to the haemagglutinin, the 'incomplete forms' of KP are balloon-like particles measuring 50–550 mμ. in the flattened state. One further possibility may be briefly mentioned, namely that the haemagglutinin may be a sub-unit, not only of the elementary particles, but also of the 'incomplete forms'. It must be pointed out, however, that in contrast to the *haemagglutinin* isolated from elementary particles, the 'incomplete forms' are incapable of protecting animals from KP infection (Table I). The significance of this difference is not yet understood. We hope that the disintegration of 'incomplete forms' will give more information on this question.

Conclusion

I think that the results presented here bring us somewhat nearer to a better understanding of the multiplication of an animal virus. It seems to me an especially important finding that the elementary particle in the form of g.-antigen includes an RNA-containing component which corresponds, not only serologically, but also chemically and physicochemically to the isolated s-antigen. This gives us some idea about the nature of the s-antigen. Certainly, further elucidation of the multiplication process will be gained by extending study of the structure from the elementary particles to the other virus-specific units, especially to the 'incomplete' and 'filamentous forms'. But a rounded picture of the multiplication process will not be achieved until the sequence and the mode of forma-

the extraction of lipid-bound phosphorus from the virus particle was therefore investigated. Secondly, the extent to which nucleic acid was extracted from the dried virus by a hot 10 per cent (w/v) sodium chloride solution was studied, the criterion adopted being the absorption at 260 mμ. of virus material (in alkali) before and after such extraction.

The validity of both approaches was assured by showing that nucleic acid was not extracted by the lipid solvents used and, in the second case, by using a digest of a nucleic acid-free protein to correct for the contribution made by the virus amino acids to the absorption at 260 mμ. (Ada and Perry, 1954)

A chloroform-methanol mixture was found to be the most efficient solvent for phospholipid extraction. The phosphorus in the defatted virus residue, when fractionated by the Schmidt-Thannhauser (1945) procedure, yielded values of 1·0 per cent RNA and 0·04 per cent DNA. All material with a maximum absorption at 260 mμ. was extracted by the 10 per cent salt solution. The extract containing the nucleic acid was analysed as follows. The indole test for deoxypentose (Ceriotti, 1952) gave a result which indicated that the DNA content of the virus was less than 0·02 per cent. In a virus of particle weight $3 \cdot 5 \times 10^8$, this may amount to a DNA fraction of mol. wt. $= 7 \times 10^4$; the mol. wt. usually assigned to DNA is about 5×10^6 (e.g. Rowen, Eden and Kahler, 1953). On this basis, DNA, if present at all, cannot be considered an integral part of the virus particle. The RNA content by u.v absorption ($\varepsilon_p = 9{,}200$) was 0·86 per cent (Ada and Perry, 1954, 1956).

The virus nucleic acid was purified by precipitation from the extract by the addition of ethanol and further analysed. The purine-bound sugar, liberated by acid hydrolysis, was isolated and identified as ribose by chromatographic analysis. The ribose was recovered to the extent of 92 per cent of that expected by theory (Ada and Gottschalk, 1956). Four bases were found following digestion with either hydrochloric or perchloric acids and separation by one- or two-dimensional chromatography. They were identified by their chromato-

RIBONUCLEIC ACID IN INFLUENZA VIRUS

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Early analyses of influenza virus purified from infected allantoic fluid (Taylor, 1944; Knight, 1947) indicated the presence of lipid, protein, nucleic acid and carbohydrate in excess of that required by the nucleic acid content. Both ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) were considered to be present, estimates in the case of RNA varying from 0 to 4·6 per cent, and ranging from 3 per cent to 0·3 per cent in the case of DNA (Taylor, 1944; Knight, 1947; Graham, 1950). Identification of the nucleic acid was largely based on colour reactions for pentoses and deoxysugars, and the amounts present were estimated from the intensity of these reactions and from the amount of phosphorus left in the dried virus after extraction with lipid solvents. Knight, however, was able to isolate from the purified virus a sample of nucleic acid which proved to be susceptible to the action of purified ribonuclease, thus establishing the presence of RNA in the virus particle.

Our work, reported here, has been concerned with the content of RNA in influenza virus and the base ratios of the viral RNA.

Identification and estimation of the virus nucleic acid

Two approaches using independent criteria were made in the present work. In view of the high phospholipid content (Taylor, 1944) it seemed possible that the high values for nucleic acid phosphorus obtained by earlier workers might be due, at least partly, to phospholipid which resisted extraction by the lipid solvents used. The effect of various solvents on

How does this result compare with the situation found with other viruses? In the case of certain plant viruses and the T2 coliphage, infection of the host cell under certain conditions results in the formation of particles which, though closely related serologically, chemically and/or structurally to the

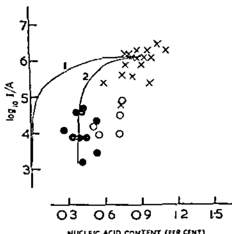


FIG. 1 Relationship between the infectivity/haemagglutinin ratio and the nucleic acid content of influenza virus

× single passage (0 05 ml, $ID_{50} = 10^{-4} - 10^{-6}$, 42 hours)
○ double passage (each 1 0 ml undiluted fluid)
● triple passage (each 1 0 ml. undiluted fluid)

For explanation of curves 1 and 2, see text.

virus particle, are non-infectious. Analyses indicate that the non-infectious component(s) contains none or only a minor fraction of the nucleic acid present in the infectious particle (Markham, 1951; Jeener and Lemoine, 1953; Rich, Dunitz and Newmark, 1955; see Epstein, 1953).

If incomplete influenza virus contained no nucleic acid and did not contribute to the infectivity titre, the relation between

graphic and spectrographic behaviour as adenine, guanine, cytosine and uracil. The presence of trace amounts of other bases cannot be excluded however (Ada, unpublished).

Throughout most of the work reported hereunder, the salt extraction method has been used for isolation and estimation of nucleic acid.

RNA as a functionally significant component of influenza virus: *the relation between infectivity* and nucleic acid content

Von Magnus (1946) and later Fazekas de St. Groth and Graham (1954) have described conditions of infection of the allantois which result in the production of "incomplete" influenza virus. It has been shown (von Magnus, 1954) that, while the haemagglutinating, enzymic and serological behaviour of incomplete virus is unchanged compared with standard virus, the incomplete virus is less infective, the degree of incompleteness being indicated by the ratio of infectivity to haemagglutinin, I/A. In this aspect of our work, the type A strain PR8 was used Standard virus was prepared by one passage of diluted inoculum, incomplete virus by two or three serial passages of undiluted inocula. The nucleic acid content of a large number of virus preparations whose I/A values ranged from 6·5 to 3·2 has been determined with the results plotted in Figure 1.

Here the value of $\log_{10}$ I/A is plotted against the nucleic acid content, the latter being estimated from the absorption at 260 mμ. of the salt extract. Despite a considerable variation, both in the infectivity of virus preparations of similar nucleic acid content and in the nucleic acid content of virus preparations of similar infectivity, it is clear that a reduction in the I/A ratio is accompanied by a decrease in nucleic acid content. In a smaller series of experiments, a similar trend was found when the I/A value was plotted against the nucleic acid content as estimated from the amount of phosphorus present in the 10 per cent salt extract (Ada and Perry, 1956).

content above a certain value, arbitrarily chosen as 1·0 per cent in Figure 2, can induce *continuing infection*, that is, are fully infectious. A parent particle with a smaller amount of nucleic acid, e.g. 0·8 per cent, may give rise to a family of particles in which the distribution of nucleic acid is more like that pictured in the second preparation (Fig. 2) where there is a larger proportion of particles which may undergo restricted multiplication. Perhaps one stage is the formation of particles which haemagglutinate but do not multiply. The important point is that only those particles which are fully infectious will contribute to the infectivity titre, as this is estimated by the process of limit dilution. The variation in the nucleic acid content of virus preparations of similar infectivity could be explained in this scheme Provided the proportion of fully infectious virus in two preparations was similar, the frequency distribution of nucleic acid contents in the remaining portions could vary markedly, resulting in different mean values.

This general interpretation would be consistent with the concept of intermediate grades of incompleteness (Burnet, Lind and Stevens, 1955), based on the observation that incomplete virus produced a higher yield of haemagglutinin in one growth cycle than would be expected from the infectivity titre.

Proportion of bases in virus nucleic acid: specific differences between A and B strains of virus

In line with earlier findings (Markham, 1953, Knight, 1954) demonstrating the specific nature of plant virus nucleic acids, the nucleic acid associated with influenza virus purified from infected allantoic fluid has been found to possess very constant properties as indicated by the proportion of the nitrogenous bases. In the case of the A strain PR8, the values obtained are presented in Table I When the nucleic acid of a

infectivity and nucleic acid content for mixtures of incomplete virus with infectious (i.e. containing 0·9 per cent nucleic acid) virus would be expressed by curve 1, Figure 1. If incomplete virus contained 0·40 per cent nucleic acid, a value slightly higher than the lowest amounts found in our experiments, the relationship would be expressed by curve 2, Figure 1. Clearly

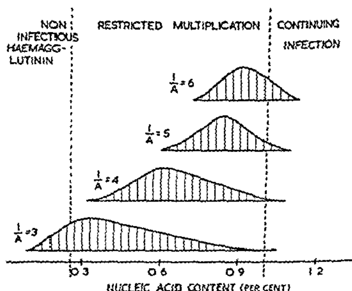


Fig. 2. Possible frequency distribution of nucleic acid in four hypothetical virus preparations whose infectivity/haemagglutinin (I/A) ratios vary from 6 to 3

neither curve, nor any that could be drawn between them, fits the experimental data, and it can be concluded that incomplete virus is not uniform with respect to nucleic acid content.

The possible frequency distribution of nucleic acid in four hypothetical virus preparations whose I/A values vary from 6 to 3 is presented in Figure 2. For simplicity, a continuous spectrum of nucleic acid content is assumed in each case.

It may be that only virus particles with a nucleic acid

values within each category are not significant ($p > 0 \cdot 1$) (Ada and Perry, 1956).

The demonstrated range of values for this ratio is thus 1·22—1·28 for A strains and 1·38—1 43 for B strains.

Is this proportion of bases characteristic of the nucleic acid in the virus particle or is it shared by other nucleic acid fractions in the infected host cell? If the RNA in an alkaline digest of the uninfected host cell is analysed, a value of about 0·6 for the ratio adenine + uracil/guanine + cytosine is found. In the infected host cell, however, there is one substance which is of particular interest in this connection. This is the soluble complement fixing antigen.

Relationship between the nucleic acid moieties in the soluble complement fixing antigens of the virus particle and of the infected host cell

One of the most intriguing phenomena associated with infection by influenza virus is the production of a "soluble" antigen which, when mixed with specific convalescent serum, will fix complement This soluble complement fixing antigen (CFA) is group specific, all A strains producing a CFA which is sharply distinct from the soluble CFA produced during a B strain infection. This cellular soluble CFA is a much smaller particle than the infective virus particle.

Recently, both Hoyle (1950) and Schafer (1955) have shown that controlled degradation of the virus particle with ethyl ether releases a soluble antigen which also reacts with specific convalescent serum. On purification, this virus soluble CFA was found to be a nucleoprotein Dr. Edney and I have tried to find out if the cellular soluble CFA is a nucleoprotein and if so, what is the relationship between the nucleic acid moieties of the two antigens?

Virus soluble CFA was prepared from an A type virus by Schafer's method The cellular soluble CFA was liberated from infected membranes by several cycles of freezing and thawing (Fulton and Isaacs, 1953), sedimented from the extract by high

Table I

Proportion of Bases in the Nucleic Acid from PR8(A) and from LEE(B) Virus

Virus	*Nucleotide composition (as moles per 100 moles nucleotide)*			
	Adenine	*Guanine*	*Cytosine*	*Uracil*
PR8(A)	23·1 ± 0·2	20·2 ± 0·5	24·0 ± 0·7	32·9 ± 0·5
LEE(B)	23·0 ± 0·2	18·3 ± 0·3	23·1 ± 0·5	35·6 ± 0·9

Elson and Chargaff (1954) have shown that ribonucleic acids from different sources may be characterized according to the value of the ratio, adenine + uracil/guanine + cytosine; when evaluated in this fashion, the two virus nucleic acids are found to differ significantly ($p < 0 \cdot 05$) *The nucleic acids of four* other A strain and two more B strain viruses were analysed and the ratios calculated with the result shown in Table II.

Table II

Values of the Ratio, Adenine + Uracil/Guanine + Cytosine of the Nucleic Acids from Five A and Three B Strains of Influenza Virus

Virus Strain		*Number of experiments*	*Adenine + uracil / Guanine + cytosine*
A	PR8	5	1·27 ± 0·02
	MEL	2	1·22 ± 0·01
	WSE	2	1·26 ± 0·01
	SWINE	2	1·24 ± 0·04
	CAM	3	1·28 ± 0·01
B	LEE	4	1·42 ± 0·04
	MIL	3	1·43 ± 0·05
	ROB	2	1·38 ± 0·03

The difference between the means of any pair of A and B viruses is significant ($p < 0 \cdot 05$); differences between the mean

base proportions of the virus nucleic acid are more irregular than and differ appreciably from those of allantoic fluid virus. This is shown in Table IV. For comparison the base proprotions of a ribonucleoprotein also present in the infected cell are tabulated as well Both the variability in the lung virus

Table IV

THE PROPORTION OF BASES IN THE NUCLEIC ACID OF VIRUS (MEL) GROWN IN THE ALLANTOIS AND IN THE CHICK EMBRYO LUNG, AND OF A NUCLEOPROTEIN ISOLATED FROM INFECTED LUNGS

Material	*Number of experiments*	*Nucleotide composition (per 100 moles nucleotide)*				$\frac{\textit{Adenine + uracil}}{\textit{Guanine + cytosine}}$
		Adenine	*Guanine*	*Cytosine*	*Uracil*	
Allantoic fluid virus	2	23 0±0 8	19 6±0 1	25 4±0 1	32 0±0 7	1 22
Lung virus	5	20 5±0 7	28 4±2 1	27 7±0 8	23 4±2 3	0 78
Nucleoprotein from infected lungs	4	20 7±0 3	33 2±0 6	27 8±0 8	18 3±0 5	0 64

values and the fact that they lie between those of the allantoic fluid virus nucleic acid and those of the host cell nucleic acid would favour the interpretation that the lung virus is contaminated with host nucleic acid. If we restrict the term "contamination" to mean the attachment of material to the surface of the virus particle, two considerations make this unlikely (1). The purification procedure includes two cycles of adsorption to and elution from red cells, a process which, being enzymic in nature, limits the amount of possible surface contamination of the virus particle (2) The nucleic

on the surface of the virus particle to an extent suggested in Table IV would result in an overall nucleic acid content in such a complex of 2–3 per cent.

Other relevant information should also be mentioned. The sedimentation diagram suggests a very wide range of particle

speed centrifugation (Ada, Donnelley and Pye, 1952) and purified further by exhaustive treatment with ethyl ether.

The two antigens were found to have comparable CF activities per mg. dry weight when tested against convalescent mouse serum obtained following infection with an A virus. They also reacted with convalescent serum to an A prime virus but not with convalescent serum to a B virus.

The results of the chemical analysis, presented in Table III, indicate that while both antigens are nucleoproteins, the RNA associated with the cellular soluble CFA differs both in amount and character from that present in the virus soluble CFA preparation.

Table III

COMPARISON OF THE NUCLEIC ACIDS OF THE SOLUBLE COMPLEMENT FIXING ANTIGENS (CFA) ISOLATED FROM THE VIRUS PARTICLE AND FROM THE INFECTED HOST CELL

Source of CFA	*Ribonucleic acid content (%)*	*Nucleotide composition (per 100 moles nucleotide)*				*Adenine + uracil / Guanine + cytosine*
		Adenine	*Guanine*	*Cytosine*	*Uracil*	
Virus	5 3	23 2	20 0	24·1	32 7	1·27
Cell	0 7	22 2	33 3	26·4	18 1	0·68

If the nucleic acid moiety is considered an intrinsic component of each of the soluble CF antigens, they must be regarded as chemically distinct compounds. Until we know something about ribonucleoproteins present in uninfected cells, however, it would be premature to attempt a further interpretation of these results.

The nucleic acid of virus grown in different tissues

We have attempted to repeat some of these observations using material from infected chick embryo lungs but, in preliminary experiments with a type A virus, have found that the

If in future experiments we can eliminate contamination by or incorporation of host cell nucleic acid, we will have to consider the possibility that only particular sections of the RNA molecule carry the characteristic virus code and that the two preparations of virus nucleic acid have only these sections in common; other sections of the molecule may vary according to the host cell.

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size with a mean sedimentation constant of 750; the sedimentation constant of A strains purified from allantoic fluid varies from 600–700. Electron microscopical examination shows very marked pleomorphism with an appreciable proportion of unduly large particles which may account for the finding that the value of the ratio, haemagglutinin/mg. dry weight is one half to one third the value of purified allantoic fluid virus.

Conclusion

We can briefly recapitulate as follows. Influenza virus convirus contains about 0 9 per cent RNA. The relationship found between the infectivity and nucleic acid content of the virus particle has been interpreted as evidence that the nucleic acid moiety plays a significant part in virus multiplication. The specific difference found between the nucleic acid base ratios of the A and B strains of virus supports the contention that the nucleic acid molecules in the virus particle are built to a special and characteristic pattern, as reflected in the base ratio This belief is strengthened by the finding that this base ratio characterizes the nucleic acid moiety of the soluble CFA isolated from the virus particle but not the nucleic acid of the soluble CFA present in the infected cell. This interpretation is compatible with the many recent observations concerning the rôle played by RNA in protein synthesis, which encourages us to believe that the RNA molecule carries coded information (e.g. *Gale and Folkes*, 1955).

It is surprising therefore to find that the proportion of bases in the nucleic acid of virus grown in the embryo chick lung and in the allantoic cavity differ. It may be suggested that during fabrication of the lung virus particle, a variable amount of host cell nucleic acid may be incorporated as well as the characteristic viral nucleic acid. Some support for such a scheme is provided by the finding that the lung virus preparation contains many large particles. It seems unlikely that such a *large discrepancy could be explained in this way* however.

different. In our work we studied viruses which are not so easily purified as the ones which can be absorbed by red cells and then eluted, and with the relatively crude preparations at our disposal we were

Ada: According to the electron microscope counts it is probably of the order of 1 10.

Bang Dr. Schafer, how many of the haemagglutinin particles may be adsorbed onto a red cell?

Schäfer · I have no idea

Bang Have the antibodies that you produced by injecting virus been tested for neutralizing activity against the virus?

and the fowl plague shown by other strains of influenza, or is it characteristic of this one strain?

DISCUSSION

Pirie: These observations of Mr Ada's are extraordinarily interesting and almost completely agree with what Bawden and I find with TMV—that there is no sedimentable material with the serological specificity of TMV and completely free from nucleic acid (Bawden, F. C., and Pirie, N. W. (1956) *J. gen. Microbiol.*, 14, 460). Many years ago we made slowly sedimenting, serologically active but relatively non-infective fractions from leaf extracts. The fractions we make now invariably contain at least one-fifth of the normal amount of nucleic acid and sometimes as much as half. This is similar to the variability that you find.

Dulbecco: What is the proportion of complete particles in the lung virus?

Ada: The I/A ratio is the same as you have with allantoic fluid virus.

Dulbecco: Is it not possible that the particularly large forms that you described were partially incomplete particles and therefore had a low RNA content?

Ada: This is possible as on the basis of the I/A ratio, only one particle in ten is infectious as is the case in allantoic fluid virus. We cannot say whether the large particles are "complete" or "incomplete" virus.

Dulbecco: How do you explain the difference between the soluble antigen which is in the cell and that which is in the virus? Wouldn't you think that the soluble antigen which is in the cell is the precursor of the one that goes into the virus?

Ada: It may not be. The cellular soluble antigen may merely represent a pattern which has been conferred on host cell protein as a result of virus infection.

Stoker: Can you account for thermal inactivation at 37° by loss of RNA? Have you examined virus after thermal inactivation?

Ada: We have tried that, but it doesn't make very much difference to the RNA content.

Stoker: Since the base ratio differs so markedly in the different parts of the egg, is the base ratio the same in incomplete virus?

Ada: Yes. The RNA of incomplete virus grown in the allantoic cavity has the same base ratio as that of standard virus.

Stoker: Does influenza B virus also change its base ratios in different tissues?

Ada: We have not as yet investigated that.

Stoker: Dr. John Smith (Smith, J. D., and Stoker, M. G. P. (1951). *Brit. J. exp. Path.*, 32, 433) has examined the DNA of some rickettsiae, and in the *R. burnetii* the base ratios were almost exactly the same as those of the DNA of the egg host except, fortunately, for 5-methylcytosine which could be used as a marker for host contamination. But we have found that other species of rickettsiae grown in the egg had entirely different DNA base proportions.

v. d. Ende: I was rather impressed with the fact that Dr. Schäfer's soluble antigen and the g-antigen which he obtained from his virus are so similar, whereas, on the other hand, Dr. Ada's soluble antigen which he gets from the cell and the soluble antigen from the virus are

I believe, used too. I think one of the strongest bits of evidence that this is not due to contamination is the fact that the material in the virus which cross-reacts is not absolutely identical with the material present

that we have not found a serological cross-reaction between host protein

enzymes in *coli* and we must postulate, on a conservative basis, about 200 more, or something of that order Each of these is an antigenetically specific protein This is an assumption, but I think we all concur on that one

antiphage activity.

Smith I was very interested that it was reported that the incomplete

virus

[illegible] Mr Ada, haven't you had other preparations of soluble antigen with a higher RNA content?

code already exists in the cell. If you infect that cell with influenza virus, it goes on making ribonucleoprotein but it is not quite the same ribonucleoprotein as it was before. But the modification may not be a very big one, and you don't necessarily need a big molecule to produce it

Dulbecco: This question that Dr Hoyle raises should be discussed in a more general form—not only animal viruses should be considered for this problem but also viruses like phages, for example. Now in phage I think that you could hardly claim this because of both the chemical evidence and the genetical evidence The chemical evidence is that the viral nucleic acid contains a base which doesn't exist in the bacterium, and the genetical evidence shows that the genetic material of the virus can be separated in functionally different units the length of which is about 10^{-5} of the viral DNA, on the basis of the recombination data of Beurer, N Franklin and others. Therefore it seems that there is just enough nucleic acid to carry the genetic information of the virus

In the case of an animal virus, of course, we don't have any of this kind of information, but I think that we should not make theories based on the lack of knowledge.

Hoyle. You must not argue by analogy between the DNA-containing viruses and an RNA-containing virus either!

Pirie: I concur very emphatically with Hoyle. The argument used by Dulbecco depends, I think, on lack of knowledge about the composition of the host cell. It is clear that the special base does not make up 1 per cent of the total base in the host cell but it is perfectly possible for it to be a minor component taking part in a normal cycle and for this cycle to be pushed into an important position when phage production starts

Sanders There is one further point that should be borne in mind. Invasion of bacteria by phage DNA is accompanied by the breakdown of the nuclear apparatus of the bacteria I don't know of any instance where this happens with the invasion of cells by an animal virus

Lwoff. It seems to me that if Dr. Hoyle's hypothesis was right, one should expect to find a serological relation between normal proteins of the cell and proteins of the virus. This is not the case.

Hoyle But are we quite sure it isn't?

Lwoff. This is certain in the case of phage.

Crick: I would like to make an analogy which might do something to reconcile these points of view, that is the case of lysogenic phage which, when it goes into the cell, in some way "attaches" itself to the DNA in the cell Now, by analogy it is possible that when the RNA virus goes into the cell there is perhaps a similar process with the cell RNA, and that the reason you can only infect certain cells is that you may have a certain "attaching step". It is true that in the lysogenic case the attaching step is a preliminary to the virus disappearing, but that may not be an important difference.

Smith: I want to take exception to one statement that has been made and that is that there is no serological cross-reaction between the virus and the host protein. In my laboratory at least five years' work has been devoted to accumulating evidence leading to the conclusion

[illegible]

tentatively have a value which is consistent with the one reported by Mr. Ada [illegible]

identical [illegible]

on rather well [illegible]

not viruses.

[illegible]

th[illegible] ye[illegible] [illegible] viruses, de-

[illegible] virus can be produced, for example, by thermal denaturation of standard virus In our experiments, we endeavoured to reduce to a minimum this possibility

Stoker· But the RNA content is not altered as in your other definition?

Hoyle Our views in Northampton about incomplete virus are very simple. We think that a standard virus is a virus in which one particle contains enough nucleic acid to infect a cell and therefore to infect the

Ada: Yes, we have purified soluble antigen from infected chick embryo lungs. The RNA content of such preparations may be as high as 7 per cent. Many preparations also contain DNA. The base ratio of the RNA is similar to that of the RNA in allantoic membrane preparations.

Dulbecco: You said that you were worried about the RNA being in

inclined, however, to agree with Dr. Dulbecco that it seems as though it is more likely that this is a stranded sort of material that only occasionally breaks apart

Another question I want to ask Dr. Schafer is this. if one allows for the obvious flattening and hence the enlargement of the diameter of the incomplete form, do you think there is any reason to believe that volumewise the incomplete form is any larger than the complete form?

Schäfer: I cannot imagine that our largest "incomplete *forms*" having a diameter of about 550 mμ can be produced by flattening of a spherical particle with 70 mμ diameter, like the elementary particle.

work that the protein content of these "incomplete forms" is very low. Can't their RNA/protein ratio be the same as that of the infectious virus particle?

Ada: We do not find any correlation between sedimentation and lipid content. For example, we prepared two batches of a B strain, ROB, from the same seed virus. As far as we could tell, the final preparations were of similar degree of purity. Their characteristics were as follows:

Batch 1. $S_{20} = 720S$, lipid = 20 per cent
Batch 2. $S_{20} = 760S$, lipid = 40 per cent

A large number of preparations of PR8 virus whose I/A values

Mr. been eter- and

tentatively have a value which is consistent with the one reported by Mr. Ada

We are a little bit disturbed by this report that the RNA's of virus

identical.

Dulbecco Is it possible that since you extract the virus from the lung tissue, you may have extracted something from the lung tissue which

on rather well.

not viruses

In our experiments, we endeavoured to reduce to a minimum this possibility.

Stoker But the RNA content is not altered as in your other definition?

Hoyle: Our views in Northampton about incomplete virus are very simple We think that a standard virus is a virus in which one particle contains enough nucleic acid to infect a cell and therefore to infect the

egg. An incomplete virus is a particle in which the total content of nucleic acid is not enough to infect the cell. Therefore, in order to infect the cell you must have more than one particle.

Now, from a consideration of mathematics, if it is necessary to infect the cell with more than one particle, the actual dose which you would have to inoculate into the allantoic sac in the fertile egg goes up by 16,000 times. Therefore, a preparation of incomplete virus appears at least one-thousandth less infective than a preparation of standard virus. But the real difference is that it has only got half as much nucleic acid

CHEMICAL INACTIVATION OF VIRUSES*

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THE question of the kinetics of chemical virus inactivation has become a problem of more than academic interest after the occurrence in the U.S.A of inoculation poliomyelitis in children vaccinated with formalin-treated virus. Since adequate information on this subject is scarce or lacking the Swedish group engaged in the production of a polio virus vaccine has considered a systematic attack on the problem necessary.†

Salk (1956) has repeatedly stated that inactivation of polio virus by formaldehyde (*F*) runs the course of a first order reaction. At the third International Poliomyelitis Conference in Rome in 1954 I pointed out that the Swedish observations did not conform with this statement (Gard, 1955) On the contrary, we had found a systematic and consistently reproducible deviation from the linear log. activity-time relationship postulated by Salk On this occasion Salk attributed the discrepancies to technical differences, particularly to the fact that the virus used in our experiments had not been pretreated by filtration and thus were to be considered insufficiently homogenized Later we confirmed our previous results in numerous experiments applying pre-filtration through sintered glass filters of varying porosities, including Corning ultra-fine tubular filters (Wesslén, Lycke, Gard and Olin, 1956). Filtration usually causes a slight reduction in virus

School

content but has had no demonstrable effect on the course of the inactivation curve.

Methods

Before reporting and discussing our observations I shall briefly describe and comment upon some technical details. The action of F upon organic matter is extremely complex. Amino and imino groups seem to be preferred reaction sites, but in addition amido, peptide and sulphydryl groups, tyrosine, tryptophan and other cyclic structures including purines and pyrimidines combine with F. Most of these reactions proceed in at least two stages. First, a fully reversible combination occurs leading to a well-defined equilibrium between free and bound F. In the next step, a more firm compound emerges by secondary methylene bridge or ring structure formation. The first phase of the reaction proceeds very rapidly; equilibrium is reached within seconds or minutes. Secondary changes develop much more slowly and seem to lead gradually to increasingly stable compounds.

In a crude tissue culture fluid the virus represents but a negligible fraction of the F-reactive material. The bulk is made up by amino acids in the fluid medium and by proteins and degradation products released from disintegrated cells. The latter factor is largely uncontrollable, and therefore the raw material for vaccine production shows not inconsiderable variations in F-combining capacity. Two factors are to be taken into account in this connection: the primary equilibrating effect upon the level of concentration of free F and the slow secondary consumption of F.

In order to minimize the effect of variations in the composition of the material we have tried to establish a buffered medium by addition of glycine, a procedure devised by Neumuller (1954). Glycine has a very high affinity to F and the reaction seems to remain fully reversible for an indefinite period of time at neutral pH. Therefore, in a mixture with competitive substances glycine will largely determine the level of the F equilibrium and, if added in excess, provide an

F bank from which gradual losses of F may be compensated and a constant concentration of free F may thus be maintained. However, the addition of large amounts of chemicals to the reaction mixture complicates the activity assay of the product. For this reason we have reached a compromise restricting the glycine concentration to 0·02 M, which is not sufficient to provide a complete stabilization, although variation is considerably reduced As a rule, F was added to a final concentration of 0 006 M. As there are no reliable methods for determination of free or active F, the reaction mixture cannot be characterized in this respect Presumably 60 to 70 per cent of the total F forms a reversibly-bound reserve.

The inactivation process was interrupted by addition of bisulphite in excess. It was found that neither reactivation nor progressive inactivation took place on storage of bisulphite-treated material

Salk has chosen 37° C for the inactivation process. At this temperature, thermoinactivation amounts to 1 to 2 log units in three days, somewhat depending upon the composition of the medium As the Salk procedure aims at an initial inactivation rate of about 6 log. units in three days it is obvious that the thermal component of the reaction contributes substantially to the net result. In order to breed F inactivation more pure we have chosen 25° C as reaction temperature, thereby reducing thermal inactivation to about 0 3 log units in 5 days.

The pH of the reaction mixture has been adjusted to and maintained at 7·0 ± 0 2.

Virus was grown in suspended fragment cultures of human embryonic skin-muscle tissue in Parker 199. For activity determinations samples of sulphite-treated reaction mixtures were dialysed against the fluid medium No loss of activity occurred during dialysis Titrations were performed in plasma clot cultures of human embryonic lung tissue Seeded cultures were observed for 12 days and results were confirmed by typing and passages of all cultures inoculated at critical dilution levels. Determinations in the activity range below

the "baseline" were based on the principles laid down by Halvorson and Ziegler (1932) and Haldane (1939). Rolling flask or rocking flask cultures were inoculated with 1·0, 10 or 25 ml. amounts and the most probable activity calculated on the assumption of a Poisson distribution of the percentage of takes.

Analysis of results

In Fig. 1, the results of 12 inactivation experiments on all three types of virus are summarized. A statistical analysis

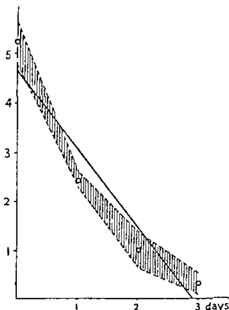


FIG. 1 Inactivation of polio virus by formaldehyde Means of twelve experiments. Shaded areas confidence interval

revealed no significant differences between individual experiments or between types, so the material was pooled. On the ordinate the mean titres are plotted on a log. scale against

time of F treatment on the abscissa. The shaded area denotes the 99·9 per cent confidence interval. The best fitting straight line does not come within the confidence limits of anyone of the four points.

Fig. 2 shows an experiment in duplicate on Type 2 with duplicate titrations on most samples. The deviation from a linear course is obvious. When plotted against log. time, on

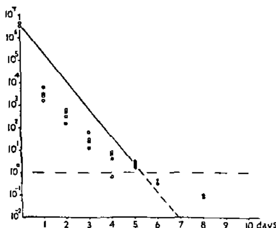


FIG 2 Inactivation of Type 2 polio virus by formaldehyde Experiment in duplicate ○ — activity of undialysed samples, ● — activity of dialysed samples

the other hand (Fig 3), the curve seems to approach a linear assymptote. The result of a statistical analysis of six such experiments is presented in Table I. The qualities analysed were the fit to a linear regression of log activity upon time

Out of six experiments two showed a significant and two a probable deviation from Salk's linear relationship In the pooled material a deviation on a very high level of significance

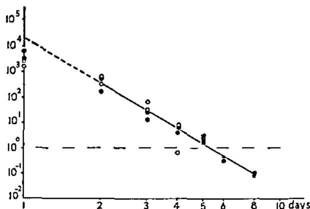


FIG. 8 Same experiment as in Figure 2 Log activity plotted against log time

was demonstrable. The fit to a linear regression upon log. time was excellent, in the individual experiments as well as in the pooled material.

Table I

PROBABILITY OF LINEAR REGRESSIONS OF ACTIVITY UPON TIME (SALK CURVE) AND LOG TIME, RESPECTIVELY. VARIANCE RATIOS MEASURE DEVIATION FROM LINEAR COURSE

Exp. No	*Salk curve*		*log. time*	
	b	*ratio*	*b*	*ratio*
I:1	— 1 42	4 97	— 6·16	0·76
I:2	— 1 30	15 60^{xxx}	— 5 97	1 13
I·3	— 1 38	6 81^{x}	— 6 41	0·43
II 1	— 1 05	10 67^{x}	— 5 80	0 42
II:2	— 1 13	1 11	— 6·31	0 24
II:3	— 1 03	21·53^{xx}	— 5·53	1·14
Mean	— 1·23	18 90^{xxx}	— 5 89	0·65

b = coefficient of regression.
Significance levels. $x = 0\ 05$, $xx = 0\ 01$, $xxx = 0\ 001$

In order to fit the experimental data, an equation describing the reaction should thus have an assymptote $\log \frac{y_o}{y} = a' + b' \log t$, y_o and y denoting activity at times 0 and t,

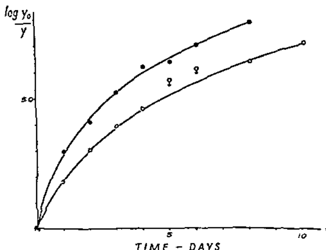


FIG 4 Inactivation of polio virus by formaldehyde Fitting of experimental data to empirical function $\log \frac{y_o}{y} = a \log (1 + bt)$ ○ — 0 006 M (3 experiments), ● — 0 007 M formaldehyde (4 experiments) Arrow indicates that definite titres were not obtained, points represent upper limits

respectively, a' and b' being constants or parameters. As a simple and plausible function fulfilling this requirement

$$\log \frac{y_o}{y} = a \log (1 + bt) \quad . \quad . \quad . \quad . \quad (1)$$

was tested. In Fig. 4, the best fitting curves of this type are compared to two sets of data from experiments with F concentrations of 0 007 M (4 experiments) and 0·006 M (3 experiments), respectively. The fit is surprisingly good. Consequently, it was considered justifiable to let equation (1) form

the basis for a discussion of the mechanism of the inactivation process.

The rate of inactivation at any given time is dependent upon the concentration of still active virus, on the concentration of F and on the specific reaction velocity constant. The reaction velocity equation obtained by derivation of (1) reads

$$\frac{dy}{dt} = -\frac{a\,b\,y}{1 + bt}$$

meaning that at least one of the three determinant factors is a function of time or a more complex function of time than assumed in a first order reaction.

Starting with y, we may assume it to enter in an order higher than one. Suppose that

$$\frac{dy}{dt} = -\,k\,F\,y^{(1+a)}$$

k denoting the reaction velocity constant. This leads to

$$\ln\frac{y_o}{y} = \frac{1}{a}\ln\left(1 + ay_o^{a}.\,kFt\right) \quad . \quad . \quad . \quad . \quad (2)$$

Equation (2) conforms to the general type of (1). As y_o enters as a parameter it requires that the inactivation be dependent upon the initial virus concentration. We have satisfied ourselves in specially designed experiments that this is not the case.

Against the background of what has been said of the reaction of F with impurities in the reaction mixture, a slowing down of the reaction rate on account of loss of F activity seems *a priori* not improbable. It is easily shown that the result of such a process can be brought to conformity with function (1) only on the specific assumption that F activity decreases on account of a polymerization process. The reaction equation then assumes the form

$$\ln\frac{y_o}{y} = \frac{k}{c}\ln\left(1 + cFt\right) \quad . \quad . \quad . \quad . \quad (3)$$

c being the reaction velocity constant of the polymerization process. This possibility was tested by addition of fresh virus to a reaction mixture in which the rate of inactivation had decreased to less than one fifth of the initial and subsequent determination of the rate by which the newly added virus was inactivated. The latter was found to correspond exactly to the original and it was thus obvious that no loss of F activity had occurred.

Remains the factor k. A dependence of k upon time alone would lead to an equation of the following type

$$\ln \frac{y_o}{y} = \frac{kF}{b} \ln (1 + bt) \quad . \quad . \quad . \quad . \quad (4)$$

in which F would enter purely as a proportionality factor. It is difficult to visualize a mechanism underlying this alternative and, besides, function (4) has been found experimentally invalid F and t seem to have the same effect on inactivation, i.e. an increase in F has the same effect as a corresponding increase in time of treatment of a mixture of the original F content. F and t should therefore be interchangeable and appear in the reaction equation together as a factor Ft.

This leads to the assumption of k as a function of both F and t. It is easily found that

$$k = \frac{k_o}{1 + bFt}$$

k_o denoting the reaction velocity coefficient at time 0. This leads to

$$\ln \frac{y_o}{y} = \frac{k_o}{b} \ln (1 + bFt) \quad . \quad . \quad . \quad (5)$$

On the condition that factors such as pH, temperature, electrolyte medium, etc. remain unchanged, the affinity between any two given substances must necessarily stay constant. In trying to interpret the apparent variation in k we have, therefore, no choice but to regard k as the mean of a number of true constants referable to a corresponding

number of reaction systems, the proportions of which are changing in the course of the reaction. In other words, the variability of k has to be regarded as indicative of an inhomogeneity of the reaction system In this case we have to consider two principally distinct possibilities.

A. Pre-existing inhomogeneity. This implies the occurrence in the starting material of a number of virus variants with different resistance to the action of F. This situation would obtain if the virus particles were to a varying extent supplied with a protective coating of extraneous material derived, for example, from the disintegrated cells. According to this line of thought, which is now being followed by Salk and the American Technical Committee (1955), inhomogeneity would appear to be largely a chance phenomenon. At least it is difficult to conceive of any mechanism by which the distribution of coating material would be regulated to such an extent as to provide a virus population of a constant make up in this respect from one experiment to the next, from type to type and from tissue cultures of different origin and composition. Under these conditions, the possibility of obtaining reproducible results would seem remote indeed. On account of the practical implications of this attempt at explanation of the phenomenon it should be thoroughly tested, however.

Homogenization of a predominantly physically inhomogeneous material may be achieved by mechanical or chemical means. So far we have only made a few attempts in this direction. As already mentioned, filtration of the material has failed completely to change the type of the inactivation curve. Moderate mechanical stirring had no effect. We have now devised a more powerful stirrer which will be tested in the near future. Tryptic digestion of the virus prior to F treatment, aimed at removing protein coats, did not cause any demonstrable change.

A pre-existing heterogeneity might also be genetically conditioned, provided that a natural dissociation of the virus into variants of different F resistance occurs. In such a case, a constant composition of a material derived from one

particular strain of virus would appear most probable. One would expect, however, to find distinctive differences in this respect between strains and types. It will not be easy to prove or disprove such an hypothesis. Lycke in our laboratories tried to breed an F-resistant variant pure by repeated selection but without success.

B. Heterogeneity as a result of the action of F. This possibility rests on the conception of the virus particle as a giant molecule possessing a very large number of F reactive sites. From the work of Miller and Stanley (1941, 1942) we know that a large proportion of such groups may be chemically substituted without impairment of the biological activity. A certain number, however, belong to vital substructures and an irreversible change in any such spot will most likely result in complete inactivation

We have to conceive of the reaction between F and virus as a gradual chemical transformation of the particle which will lead to inactivation only when a vital site becomes involved. It is highly probable that such a gradual transformation will be associated with a change in chemical reactivity. It is well known, for instance, that comparatively small structural changes in amino acid molecules may produce major changes in their F affinity. Thus, the alterations in steric configurations and charge, accompanying the tanning action of F, will probably produce reaction kinetic inhomogeneity in an originally homogeneous virus population. Such a process would be governed by specific chemical laws and, consequently, reproduce itself in repeated experiments.

In order to gain some insight into the mathematical consequences of such an hypothesis I have constructed a radically simplified virus model. Suppose that each particle carries m chemically identical reactive sites, pm of which represent vital spots, while qm are biologically unimportant. At time t, a certain number of substitutions of reaction sites will have occurred and a series of chemically transformed virus particles will have appeared. The concentration of each category will be denoted as y_m, y_{m-1}, etc., indexed according

to the number of intact reaction sites. The "concentration" of reactive sites will then be $m.y_m$, $(m-1)\,y_{m-1}$, etc. Let k_o be the reaction velocity constant of the intact virus particle and k_1, k_2, etc., denote the corresponding constants of the successive transformation products. If we put $k_o.m = k_m$, $k_1(m-1) = k_{m-1}$, etc., the stepwise transformation of the virus will be described by the following series of differential equations:

$$\frac{dy_m}{dt} = -k_m F y_m$$

$$\frac{dy_{m-1}}{dt} = k_m F y_m - k_{m-1} F y_{m-1}$$

. . . .

$$\frac{dy_{m-n}}{dt} = k_{m-n+1} F y_{m-n+1} - k_{m-n} F y_{m-n}$$

. . . .

After integration these equations lead to the following solutions, Y denoting initial activity

$$\frac{y_m}{Y} = e^{-k_m Ft}$$

$$\frac{y_{m-1}}{Y} = k_m\left(\frac{e^{-k_{m-1}Ft}}{k_m - k_{m-1}} - \frac{e^{-k_m Ft}}{k_m - k_{m-1}}\right)$$

. . . .

$$\frac{y_{m-n}}{Y} = k_m \dots k_{m-n+1} \sum_{r=o}^{r=n} (-1)^r \frac{e^{-k_{m-n+r}Ft}}{(k_m - k_{m-n+r}) \dots (k_{m-n+r+1} - k_{m-n+r}) \times (k_{m-n+r} - k_{m-n+r-1}) \dots (k_{m-n+r} - k_{m-n})}$$

. . . .

On the present assumption of chemical identity of vital

substitutions will have retained activity can be calculated to $\frac{qm(qm-1)(qm-2)\ldots(qm-n+1)}{m(m-1)(m-2)\ldots(m-n+1)}$. The remaining activity will correspond to the sum total of the fractions of active particles of each category

$$y = y_m + \frac{qm}{m} y_{m-1} + \ldots + \frac{qm!}{(qm-n)!} \cdot \frac{(m-n)!}{m!} y_{m-n} + \ldots (6)$$

y_m, y_{m-1}, etc., corresponding to the functions described above.

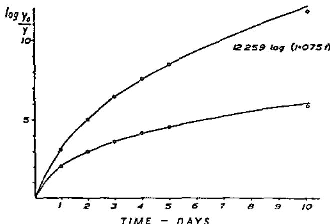


FIG. 5 Tracings of theoretical inactivation curves, function (6) (see text) Parameters $m = 100$, $p = 0.1$ (upper curve), $p = 0.05$ (lower curve). ○ — empirical function (1)

The characteristics of this complex function are not immediately clear. Therefore, I have traced the curve for some specific values of its parameters. On the arbitrary assumption that the F reactivity be proportionate to the reactive surface, curves of the type depicted in Fig. 5 were obtained. The open circles refer to function (1) fitted to the first part of the curve. In general, function (6) has a linear assymptote with a slope identical to that of the last partial reaction, i.e. the transformation of y_{pm} into y_{pm-1}. The larger the parameter m, the

longer is the curving part of function (6). The curvature is determined by the parameter p and by the sequence of reaction velocity coefficients k. Function (1) represents a good approximation of (6) and better the larger the parameter m. In Fig. 5 ($m = 100$, $p = 0{\cdot}1$) the difference between the two functions amounts to about $0{\cdot}01$ log. units at levels up to 8 log. units after which it increases slowly. At the level of 12 log. units it is still only $0{\cdot}3$ log. units. It is obvious that function (6) may describe the actual findings equally well as function (1).

The fitting of function (6) to the observed data should not be interpreted as an attempt to prove that this is the true mathematical explanation of the experimental facts. No doubt a large number of functions could be found equally well fitting. It shows, however, that the findings are not inconsistent with the theory on which function (6) is based and that consequently, the former may be adopted for the time being as a working hypothesis. Its ultimate validity has to be tested by the corollaries derived from it.

The present situation may be summarized as follows. All seem to agree on one point: that deviations of the inactivation process from the course of a first order reaction occur. According to Salk and the Technical Committee these deviations are caused by physical inhomogeneity of the material, conditioned by chance and remediable by mechanical homogenization, e.g. repeated filtration. According to the present theory, inhomogeneity is an unavoidable result of the chemical treatment, a phenomenon linked to and forming an essential part of the inactivation process as such.

Discussion

The most important corollary of our present working [illegible] its general applicability. Its principle is nothing [illegible] ‚tem of polio virus and [illegible] y virus and all types of chemical agents. It should, in fact, include all reactions

between multilocular large molecules and simple chemical substances.

Studies on the kinetics of virus inactivation to be found in the literature are mainly concerned with the action of heat or irradiation. Some data bearing upon chemical inactivation have been published, however, mostly on plant viruses. According to Fischer and Lauffer (1949) "it has been shown

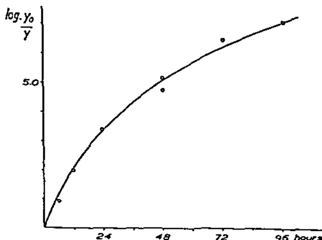


FIG 6 Inactivation of tobacco mosaic virus by formaldehyde (data from Ross and Stanley, 1938) compared to empirical function (1).

that formaldehyde-inactivation of tobacco mosaic virus, in a

hours . . . the rate of inactivation follows roughly that of a monomolecular reaction. However, a much greater length of time is required for complete inactivation than would be predicted on such a basis."

longer is the curving part of function (6). The curvature is determined by the parameter p and by the sequence of reaction velocity coefficients k. Function (1) represents a good approximation of (6) and better the larger the parameter m. In Fig. 5 ($m = 100$, $p = 0 \cdot 1$) the difference between the two functions amounts to about $0 \cdot 01$ log. units at levels up to 8 log. units after which it increases slowly. At the level of 12 log. units it is still only $0 \cdot 3$ log. units. It is obvious that function (6) may describe the actual findings equally well as function (1).

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Discussion

The most important corollary of our present working [illegible] -plicability. Its principle is nothing [illegible] -stem of polio virus and [illegible] y virus and all types of chemical agents. *It should, in fact,* include all reactions

ment with a first order reaction over a range of approximately 1·5 log. units.

Among animal viruses, that of influenza has been the preferred target. In a series of papers Lauffer and associates have dealt with inactivation of influenza A virus. Destruction of the haemagglutinin by urea was followed by Lauffer and Carnelly (1945) and later by Lauffer and Wheatley (1951). A clear-cut deviation from the first order was observed, first described as a reaction of a higher order (3/2), later interpreted as indicating heterogeneity of the virus. The authors

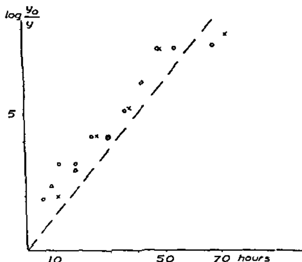


FIG. 8 Inactivation of influenza virus by formaldehyde.

assume two independent [illegible]

mental data is too wide to make a goodness-of-fit analysis profitable, it may be stated that the observation is equally well explained by a continuous change in the reaction rate, as required by our present working hypothesis

a range of 7 powers of ten. The curve represents function (1). The goodness of fit seems to be quite satisfactory. In Fig. 7, Ross and Stanley's results are compared with ours. In this representation $\log bt$ was chosen as the independent variable, $a \log (1 + bt)$ or $\log \frac{Y}{y}$ as the dependent. The curve corresponds to the parameter $a = 8$. The agreement is remarkable and

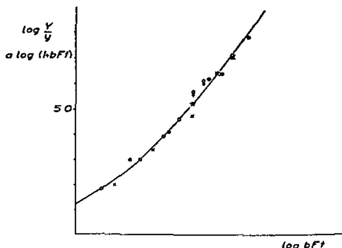


FIG. 7. Comparison of inactivation by formaldehyde of polio virus (O, ●) and tobacco mosaic virus (×); curve function (1)

indicates strongly that the reactions are governed by a common law.

From the different studies on tobacco mosaic virus it is obvious that conclusions concerning the type of reaction should not be drawn from observations confined to a narrow activity range. Unfortunately, several kinetic studies on [illegible]

denaturation of TMV by urea, in which he found a good agree-

homogeneous strain of virus, as well as the consistent reproducibility and apparent universal applicability of the phenomenon, speak decidedly against the first and in favour of the second alternative. A simple empirical equation describing the process has been found and an attempt to develop a theoretical function has been made.

The phenomena described are of immediate practical importance for the production of a formalin-treated polio virus vaccine. By establishment of the laws governing the reaction it would be possible to predict the level of activity at any time during the processing, a prerequisite of the production of a well-defined preparation of a constant quality

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DISCUSSION

Smith We have been interested for some time in the question of the homogeneity or inhomogeneity of virus preparations, and from the results of our work we have concluded that, at any rate in the case of some viruses, they are ... to definitely not homogeneous ...

Inactivation of influenza A virus by formaldehyde was studied by Lauffer and Wheatley (1949) who conclude that the reaction is of the first order. As it seemed possible that a study of a wider activity range might lead to a different conclusion, we have decided to repeat and extend Lauffer's experiments, using tissue cultures of chick chorioallantoic membranes for activity titrations as well as for production of virus. Inactivation has been carried out in the same medium as used for polio virus and at icebox temperature. Fig. 8 shows the results of some preliminary experiments. A deviation from linearity seems probable but a detailed analysis at the present stage is hardly justified.

Summary and conclusions

The rate of inactivation of polio virus by formaldehyde *seems not to remain constant but to decrease continually in* the course of the process. It has been shown that the decrease in reaction rate is not caused by a loss of formaldehyde in the reaction mixture. Furthermore, the complete absence of virus concentration dependence of the reaction rate proves that the phenomenon cannot be explained as a reaction of a higher order. Thus agglutination or aggregation of virus seems to play no essential part. As the only remaining possibility the phenomenon must be interpreted as evidence of heterogeneity of the virus.

If so, two alternative theories have to be considered. First, the virus particles in the starting material might be to a varying extent protected against the action of formaldehyde by coating of extraneous material or they may be genetically heterogeneous in terms of formaldehyde resistance. The

The failure to achieve homogenization by mechanical and chemical means, and the failure to segregate a genetically

are of equal concentration; the elution behaviour of each preparation is then studied.

[illegible]

is, [illegible]
elu [illegible]
60 [illegible]

on the cells and will not elute, and in order to obtain that at the end of an hour, we stripped it off the red cells with RDE. This virus represents

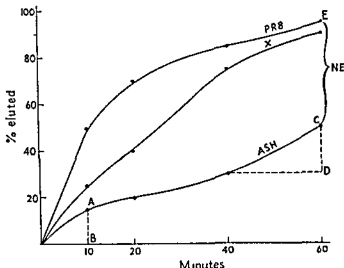


FIG 1 (Smith) Elution curves for various strains of viruses.

[illegible]

[illegible]

easily calculated. Amounts then eluted at different times can be determined on samples taken at intervals and can be expressed as percentages

, 40,

like

d in

t up

you

get

practically 100 per cent of the virus off.

TABLE I

mins.	PR8 %	X %	ASH %	NE %
10	50	25	15	0
20	70	40	20	0
40	85	75	30	0
60	95	90	50	0

Now, other strains give quite a different sort of elution curve. Many

in Figure 1.

seen in the figure this stage has not yet been reached on day 1; the points indicate a deviation in this region from the linear course in accordance with the requirements of the theoretical functions depicted in Figure 7.

Andrewes: In your third figure, is this line going to hit the ground somewhere near 10 days and does that mean that in the process of [illegible]

inactivation curve In Sweden we have settled for the requirement that the finished product should contain not more than one ID_{50} per 10^4 ml. In order to fulfil this requirement we have to extend the time of treatment to between 30 and 60 days at 25° C

Williams: And what happens to the antigenicity after that treatment?

Gard: There is no significant decrease in antigenicity at 25° C during that time.

Knight: The complicated course of the kinetics suggested by Dr [illegible]

followed by cross-linking reactions between aminomethylol groups and amide and possibly other groups. Then the formaldehyde would eventually get into the interior of the molecule and, again as Fraenkel-Conrat has shown, there appears to be a definite reaction of formaldehyde with nucleic acid itself. However, reaction with the nucleic acid would probably be impeded [illegible]

so that they are equal in this respect. As regards their adsorbability to the cell, they both adsorb and the same proportion is taken up in each case, so that presumably they have got the same amount of receptor substance.

TABLE II

	REV		NEV
Haemagglutination	+	=	+
Adsorbability	+	=	+
Elutability	+		0
Infectivity	+	=	+ (within experimental error)
Toxicity	? ±		+

With regard to infectivity, as far as we can determine, within the limits of experimental error, both are equally infective.

Lastly, we studied the toxicity, because all this study was designed really to see whether the toxicity was a function of enzymic activity. Rapid-eluting virus shows some toxicity, it is not pure anyhow, but as far as we can determine we can quote ± for toxicity The interesting thing is that the NEV is certainly as toxic as the REV and most probably even more toxic Therefore, in an allantoic fluid preparation you can split the virus, I think, showing quite clearly that from the start it is non-homogeneous, which is one of the possibilities that Dr. Gard mentioned, though his inhomogeneity might be due to the formaldehyde treatment as he went along But here we have got a virus which is wholly infective and behaves in all respects like the other except with regard to this enzymic activity.

Isaacs Are you not just separating filaments from spheres? Wouldn't filaments adsorb on to red cells and elute more slowly?

Smith No. This has been done with strains that don't show any appreciable proportion of filaments

Dulbecco On sub-cultivation of the fractions, did the difference persist in the progeny, or was the progeny just like the original parent?

Smith That is something which hasn't been done yet, but it is the obvious thing to find out It will have to be done by limit dilution of each of these sorts of virus But against the idea of it being a genetic difference, I think it is a fact that quite obviously there is this inhomogeneity of the same type on all the strains that you examine, because even a very rapid-eluting virus like PR8 still shows a progressive elution curve; it doesn't all come off at the same time. Every type of virus examined does show this inhomogeneity, so that if it [illegible] difference I think it is very remarkable to get [illegible] thing occurring with all strains

Williams: Dr. Gard [illegible]

[illegible] had

[illegible] plotted on a log-log. scale

[illegible] straight course from day 2 onwards. As clearly

[illegible]

QUANTITATIVE ASPECTS OF VIRUS GROWTH IN CULTIVATED ANIMAL CELLS

R. Dulbecco

California Institute of Technology, Pasadena, California

Two outstanding developments of virology have recently occurred. These are: (1) the demonstration that the genetic properties of viruses are determined by their nucleic acid, and (2) that in some viruses, such as bacteriophages, this rôle is performed by the desoxyribonucleic acid (DNA), and in others, such as plant viruses, by the ribonucleic acid (RNA). Animal viruses are known to contain in some cases DNA, in others RNA; for them a direct demonstration that the nucleic acid determines the genetic properties is lacking, but there is little doubt that this is the case.

The fact that two different substances are involved in the transmission of heredity may have far reaching implications, particularly since the structure of RNA can be believed to differ considerably from that of DNA. From an experimental point of view the problem is to determine which kind of correlation exists between the chemistry and the functional activity of the genetic material in the two cases.

To study this correlation in an RNA virus, a model system has to be found. This presents some difficulties. In fact, plant viruses, which are well suited for chemical work, are a poor material for biological work; animal viruses, which are becoming an excellent object for quantitative biological studies, are not so well suited for chemical studies. As a whole, however, animal viruses seem to be much preferable, because their chemical shortcomings—such as difficulty in providing large quantities of virus, difficulty of purifications—are really not essential.

Among the animal viruses containing RNA—and no DNA beyond reasonable doubt—are the poliomyelitis viruses

was reproduced. Thus, we failed completely to breed a resistant variant pure or even to find any indication of genetic heterogeneity in this respect.

Dulbecco: Is it possible that there is a reactivation phenomenon that produces the curvature?

Gard: We interrupt formaldehyde inactivation by addition of bisulphite. We have tested material, thus treated, immediately and after varying periods of storage under different conditions. We have never observed any reactivation and when a loss of activity occurred, it always equalled that of a non-treated control material. Thus, no progressive reaction in either direction seems to take place after interruption of the inactivation process with the aid of bisulphite.

min^{-1} at 50° C under certain standard conditions of pH and ionic environment. The virus is also fully pathogenic for the monkey.

From this wild type stock a first mutant type was isolated, which will be designated *d* (delayed). The plaques of this line are indistinguishable from those of the wild type at pH 7·4, but appear two days later at pH 6·8. The difference between the plaque types on acid plates is such that in a mixture of *d* and d^+ (wild type) particles in which the ratio d/d^+ may be as high as 10^5, the number of d^+ particles can be counted with a efficiency of about 50 per cent. The *d* and d^+ types can be very clearly distinguished on acid plates by a spot test. The *d* line has a greatly reduced pathogenicity for the nervous system of the monkey. Whether this reduced pathogenicity and the *d* change are due to the same mutation has not yet been established. The mutation frequency from d^+ to *d* cannot be determined for the time being, since selective procedures for the *d* phenotype are not available.

The *d* virus gives rise to mutants of the d^+ type. A *d* stock grown from a single particle contains a proportion of about 10^{-4} d^+ particles. These are detected by plating on acid medium; therefore the question may arise whether they are induced by the acid environment. To clarify this point a fluctuation test (Luria and Delbruck, 1943) was carried out. One hundred well isolated plaques were picked up from platings of the *d* stock and tested for the *d* character; they all turned out to be *d* in type. Stocks of the same titre were grown from each plaque, and the proportion of d^+ mutants in each stock was determined. Although the experiment is not yet finished, the fluctuations in the proportions of d^+ particles in the various plaque stocks so far observed suggest that the mutants were produced during the growth of the stock at pH 7·4 and not at the moment of the testing on acid medium. The estimated mutation rate from *d* to d^+ in these experiments is of the order of 10^{-6}. The heat resistance of the d^+ mutants is similar to that of the wild type virus; the pathogenicity of the mutants for the monkey has not yet been determined.

(Schwerdt and Schaffer 1955), the influenza viruses (Ada and Perry, 1954, 1955), the fowl plague virus (Zillig *et al.*, 1955), the Newcastle disease virus (Franklin and Rubin, 1956, personal communication). The poliomyelitis virus and the Newcastle disease virus are particularly suited for quantitative biological work: they can be obtained with any degree of genetical purity by plaque isolation (Dulbecco and Vogt, 1954); they can be grown in genetically pure cell strains, obtained from single cells (Puck and Marcus, 1955); the proportion of virus-yielding cells in infected populations can be accurately determined (Dulbecco and Vogt, 1955); and the yield of single infected cells can be easily examined (Lwoff *et al.*, 1955).

Poliomyelitis viruses and Newcastle disease virus seem therefore to constitute the best material for a geneticochemical study of the system constituted by an RNA virus and its host cell.

In this communication an attempt will be made to define in a preliminary and admittedly rather speculative way the geneticochemical properties of such a system; this definition will be based on experimental work of our group, of genetical, biophysical and biochemical nature.

Let us first consider some genetic aspects of the problem. This will be essentially a study of the mutability of poliomyelitis virus Type 1 (Dulbecco and Vogt, 1956, unpublished).

Several types of mutants were obtained in lines of poliomyelitis virus which had been purified through repeated plaque passages and had certainly originated from a single virus particle (Dulbecco and Vogt, 1955). These mutations involve changes in pathogenicity, in plaque types and in susceptibility to inactivation by heat.

The starting material for this work was a pure stock of polio virus Type 1 (Brunhilde, wild type). The virus in this stock gives rise to plaques that on monkey kidney monolayers develop optimally (in size and time of appearance) at pH 7·4 and develop nearly as well at pH 6·8; this virus is heat sensitive in the sense that it is inactivated at a rate of 0·29

finding for the properties of the genetically functional RNA of the poliomyelitis virus depend on the rôle of the viral nucleic acid in the multiplication of the virus We must here consider two points: (1) Genetic stability. This has two important implications. On the one hand it is very likely that the viral nucleic acid has a corresponding chemical stability; on the other the genetic material of the virus cannot be polyploid, i.e. made up of several freely assorting homologous chromosomes—as seen particularly from the stability of the various types in the variation $d^+ \rightarrow d \rightarrow d^+$. (2) Mutability. RNA and DNA viruses have a similar mutability, a remarkable fact in view of their chemical differences This similarity suggests a common mechanism of mutation. Since it is likely that mutations occur during duplication, one may think either that RNA and DNA duplicate by an identical mechanism, or that there is a common intermediate during the duplication of the two nucleic acids. For example, an RNA–DNA–RNA cycle, or a cycle involving proteins is conceivable.

We shall now turn to a second point, i e to some aspects of the intracellular virus growth, and particularly to the state of the virus during multiplication. This has been studied by following the changes of the sensitivity to radiations of the virus-cell complexes at various stages of development, according to the procedure applied by Luria and Latarjet (1947) to bacteriophage work. The experiments were carried out with poliomyelitis virus, by using ultraviolet light (Dulbecco and Vogt, 1955; 1956, unpublished). Survival curves of the complexes were obtained at various times during the latent period and during the period of virus release.

The remarkable result of these experiments is that the survival curves remained of nearly single hit type throughout the growth cycle of the virus The slope of the survival curve decreased progressively after infection, reaching a minimum value—corresponding to 1/9 of that for free virus—4 hours after infection, and remaining constant thereafter.

These experiments show that the extracellular virus is not the multiplying entity; if it were so, multiple hit curves with a

From the wild type stock a second mutant was isolated. It is a heat resistant mutant which was selected by successive steps involving heating at 50° C under standard conditions, followed by purification through plaque passages. The mutant line is inactivated by heat at a rate six times smaller than that of the wild type under identical conditions. This line is designated as t, as contrasted with the wild type, designated as t^+.

The following evidence suggests that the t character may be due to more than one mutation. A virus type, called t_1, whose heat resistance is intermediate between that of the wild type and that of the t mutant, is present in the t^+ stock in a proportion of about 10^{-5}. It is possible that during the isolation of the highly resistant mutant the t_1 type was selected first, and that out of this type the highly resistant type arose by a second mutation. The highly resistant stock may therefore be a double mutant t_1t_2, in which the two mutations have an additive phenotypic effect, like some h mutations in bacteriophages (Fraser and Dulbecco, 1953). To test whether it was a double mutant, the highly resistant virus was crossed to the wild type. In the progeny of this cross particles of intermediate resistance were found in a higher proportion than in the parental t^+ stock. This result suggests the multiple nature of the highly resistant mutant, which we shall therefore designate as t_1t_2. The t_1t_2 mutant is equal to the wild type in plaque character and in neuropathogenicity for monkeys.

The study of mutability has therefore yielded the following results: (1) spontaneous mutations (i.e. not induced by the selective factor by which they are detected) occur in the poliomyelitis virus; (2) the frequency of the $d \rightarrow d^+$ and of the $t^+ \rightarrow t_1$ mutation is of the order of 10^{-6}; (3) the two mutations [illegible] (4) the mutant character

[illegible] the true genetic changes are observed.

These results are similar to those obtained by studying a DNA virus, such as a bacteriophage. The implications of this

of this type would be established if the virus were inactivated by a tissue-specific antibody; on the contrary, the simple attachment of this antibody to the virus may be much less significant, since it may be due to the presence of contaminating host protein on the virus surface. Inactivation of viruses by host-specific antibody has been claimed only for some tumour viruses. This problem has been recently re-examined by Rubin (1956, personal communication) for the Rous sarcoma virus by using precise quantitative techniques; it could be proved that the virus is not inactivated to any extent by host-specific antibody. This antibody was found to inhibit the multiplication of the Rous sarcoma cells induced by the virus. From this study it appears likely that the effect formerly observed in this and other systems (Eckert *et al.*, 1955) in *in vivo* protection tests was a pseudoinactivation, deriving from the inhibition of the multiplication of the tumour cells.

Thus, on the basis of the available evidence, the information of the virus appears to be independent of that of the host cell.

In conclusion, the following hypothesis can be presented as a consequence of the experimental results which have been discussed The viral RNA behaves as an haploid genetic system; it carries into the cell a specific genetic information, which remains unchanged through numerous duplications. The RNA multiplication may involve an intermediate, which would also be present in the multiplication of DNA; it is an urgent problem to find out whether such intermediate exists and to characterize it. The information of the RNA of the viral progeny is independent of that of the cell Therefore the virus cannot be considered as a partly modified component of the cell. On the other hand, it appears that the virus is not an autonomous organism within the cell: the evolution of the radiosensitivity of the virus-cell complex and the concurrent metabolic changes suggest that virus components and cellular components become integrated for virus multiplication. As soon as this integration has become established, the whole cell may be considered as a single virus-producing unit.

final slope equal to that observed in the inactivation of the free virus should be obtained. The particular behaviour of the curves cannot be easily explained; however, an hypothesis advanced by Epstein (1956, personal communication) to explain the result of intracellular irradiation of bacteriophages can be used to account for the main findings. Epstein suggested that the survival curves of the virus-cell complexes reflect the inactivation of cellular centres involved in virus reproduction. As a development of this hypothesis we can interpret our findings as follows: (1) animal cells have one or two centres, which become active in virus reproduction after combination with an important component of the infecting virus particle; thus its initial radiosensitivity will be related to that of the extracellular virus. Later the resistance may change—generally it increases—due to a progressively increasing dispensability, or to stabilization of the virus component connected with the centre. (2) The radiosensitivity of the duplicating components of the virus is much smaller than that of the free virus and that of the activated cellular centre, so that the number of the duplicating units in the cell cannot be counted.

In this hypothesis the centre in question may be a key control of metabolic activity, as suggested by the occurrence of the following metabolic changes in virus-infected animal cells (Cooper, 1956; Turco, 1956; personal communications).

In chicken embryo cells infected by the virus of vesicular stomatitis, the uptake of ^{32}P from the medium is markedly reduced in comparison with the non-infected controls, beginning at the end of the latent period (3 hours after infection). At the same time the ratio of RNA nucleotides in the cytoplasmic fractions starts to change, indicating a change in the proportions of various RNAs of the cell.

[illegible] analysed is the relationship between the

between the *virus protein having specific function in infection* and *the proteins of the host cells*. *A convincing relationship*

of this type would be established if the virus were inactivated by a tissue-specific antibody; on the contrary, the simple attachment of this antibody to the virus may be much less significant, since it may be due to the presence of contaminating host protein on the virus surface. Inactivation of viruses by host-specific antibody has been claimed only for some tumour viruses. This problem has been recently re-examined by Rubin (1956, personal communication) for the Rous sarcoma virus by using precise quantitative techniques; it could be proved that the virus is not inactivated to any extent by host-specific antibody. This antibody was found to inhibit the multiplication of the Rous sarcoma cells induced by the virus. From this study it appears likely that the effect formerly observed in this and other systems (Eckert *et al.*, 1955) in *in vivo* protection tests was a pseudoinactivation, deriving from the inhibition of the multiplication of the tumour cells.

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DISCUSSION

Dulbecco No.

Sanders: Is there any possibility that this centre is in fact in the cell

justify the observed change.

the first curve changes slope in the lower part; however, I really don't think that the virus was free intracellularly because the resistance has already increased much beyond what it should have increased just by screening.

[illegible]

Dulbecco: No, it is not that. The point is that the original idea in determining these curves was that one should be able to count the number of virus precursors present in the cells. But the behaviour of the curves is entirely different from expectation since the curves are always more or less of single hit type during the growth cycle of the virus. This is a great mystery. There should have been a very great multiplicity of the curves.

Bang: This could be unravelling of DNA or something like that.

Dulbecco: But why doesn't the multiplicity of the curves appear? There should be very different curves, and this is where this centre [illegible]

this type of curve?

Dulbecco: I don't think so. If there is more than one, the distance should not matter really.

[illegible]

Dulbecco: It seems to be very general.

[illegible]

Smith: Dr. Dulbecco, why do you attribute any particular significance to the neutralizing antibodies? If I understood correctly, you said that antibodies produced to the host cell material w[illegible] that the host material is part of the [illegible] not if they react with the virus [illegible] like complement fixation and sc[illegible] that if you could get an antibody [illegible] react with the typhoid bacillus fl[illegible] part of the bacterium at all? B[illegible] neutralize or stop the infectivity c[illegible]

Dulbecco: The reasoning is rath[illegible]plicated; this is based on a great deal of work which has been done in our laboratory. It seems unlikely that the mechanism of inactivation of the virus by antibody is due simply to a kind of steric hindrance, Rubin and Franklin (unpublished) have shown that the virus can be adsorbed to the cells even if it is inactivated, but the penetration at a certain point is stop[illegible] also likely that the virus can be inactiv[illegible] body to a part different from th[illegible] For these reasons, we think th[illegible] body consists in some modificat[illegible] structure of the virus, and that therefore when the neutralization occurs, the antibody must combine with a protein which is an essential part of the virus particle. Now, it is well known in the case of influenza that the host-specific antibody does combine with the virus particle, but it does not prevent the infection, in other words, it does not neutralize. So, it is much more dubious whether the simple combination of antibody with virus involves an essential viral constituent; it may involve an adsorbed impurity, but it is likely that neutralization always involves an essential constituent

Smith· That isn't really true generally, is it, because there are many examples, including influenza, where mere attachment to a surface component will neutralize infectivity? You don't need to have a reaction with an essential internal substance at all

Dulbecco· But these are virus-specific antibodies. Of course, I think that the neutralization is always a surface phenomenon, but it depends on which kind of phenomenon.

Hoyle. What you are say[illegible] is not [illegible]

[illegible] Dulbecco's interpretation of neutralizing antibodies is a bit too sweeping. After all, there are neutralizing antibodies which definitely neutralize viruses and that reaction is a reversible one for a long time It is quite true that th[illegible] reversible probably goes on to [illegible]

antibody used.

THE MULTIPLICATION OF ANIMAL VIRUSES

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THE behaviour of viruses within their host cells can most profitably be studied under conditions where a known number of susceptible cells can be exposed simultaneously to a known number of fully infectious virus particles and the resulting events followed quantitatively. For a long time these conditions have most easily been fulfilled in the case of bacterial viruses, which have consequently become a model system for the study of virus multiplication as a whole. However, it is now doubtful whether facts derived from the study of bacterial virus replication can be transferred directly to other viruses without qualification. For example, the high DNA content of most bacterial viruses and the specialized 'head' and 'tail' structure of the extracellular infective phase set them apart from the animal viruses, where the extracellular particles are simple spheres or polyhedra and contain RNA rather than DNA. Animal viruses themselves may show as much diversity in their virus-cell relationships as any other large heterogeneous group of micro-organisms.

On evidence from the study of bacteriophage, the chain of events which occurs when a susceptible cell is infected by a virus can be divided formally into a number of phases.

There follows a period during which the virus can no longer be detected by the available techniques of measurement, and during which the processes of multiplication are initiated by a 'vegetative' form (*eclipse phase*). Later, new virus particles, judged 'mature' by their possession of the infectivity and

antigenicity common to the extracellular form, appear within the infected cells, and are then liberated into the environment. In this paper I shall use the same type of formal scheme to describe the multiplication of animal viruses, but without prejudice as to its ultimate validity. In this way it will be easier to make comparisons between the behaviour of the different agents.

Adsorption and penetration of infecting virus

Compared with the more tenuous membranes surrounding animal cells, the bacterial cell wall is a rigid and complicated structure. Bacterial viruses consequently require a highly specialized mechanism for introducing their hereditary material into the cell interior. This is achieved by the differentiation of the exterior coating of the phage particle into a disposable microsyringe, whose only function is to inject the phage DNA into the interior of the bacterium Phage particles thus have one site only, the tail, by which they can become meaningfully attached to host cells. By contrast, many animal viruses must have more than one place by which they can become attached to host cells, since they can cause red blood cells to agglutinate. More is known of the process of virus attachment for viruses of the influenza group than for any other viruses. Although they do not multiply in erythrocytes, the interaction between such viruses and suitable red cells closely resembles their reactions with the surface of other cells in which they *can* multiply, such as those of the chick chorioallantoic membrane and the mouse or ferret lung epithelium.

Like the adsorption of bacterial viruses, the primary attachment of influenza virus to the red cell is markedly dependent on salt concentration, and has a low temperature coefficient. The number of 'spots' on a human or chicken red cell at which virus can become attached is uncertain, but may be as low as 300 (Sagik, Puck, and Levine, 1955); other authors, using different methods, give a much higher estimate

(Dawson and Elford, 1949). Attachment of the influenza virus to the surface of a red cell can initiate a spreading disturbance which leads to the destruction of receptor sites and the elution of virus. The enzymic action of between 3 and 5 virus particles per red cell can destroy all the receptor material within 24 hours at 37° C. Paradoxically, while the mucinolytic enzyme of influenza virus is undoubtedly concerned in the primary attachment between virus and cell, it does not seem to be involved in cell penetration. For example, treatment of bacteriophage particles with cell-wall material triggers off a mechanism in the phage tail and results in the ejaculation of the DNA charge in the head of the particle (Jesaitis and Goebel, 1953); adsorption of influenza virus on red cells does not liberate nucleic acid, and the virus can elute. On the chorioallantoic membrane similar receptors must be involved, since attachment of virus to cells can be prevented by treating the cells with RDE, which also renders red cells inagglutinable. However, virus attached to chorioallantoic membrane cells does not elute, but soon disappears within the cells. This is shown by the fact that virus attached to cells can, in the early stages, be removed by RDE and infection prevented. No protein 'overcoat' is left behind at the cell surface as in the case of phage. Penetration of influenza virus into cells is probably a process mediated by the cells themselves, since even virus heated to destroy the mucinolytic enzyme is taken up by susceptible cells (Isaacs and Edney, 1951). Moreover, treatment of the cells with the antimetabolite AMPS (α-amino-α-p-methoxyphenylmethane sulphonic acid) blocks penetration, since in its presence the virus does not get in past the stage at which it is still removable by vigorous washing or RDE treatment (Ackermann and Massaab, 1954). The virus receptors themselves, however, are unaffected by treatment with this substance. Simple washing away of the AMPS allows the infectious process to resume and go to completion. The exact part played by influenza virus mucinase in cell invasion thus remains obscure; perhaps it is involved in eroding the mucus overlying the epithelial cells of the respira-

tory surfaces so that the virus can make contact with the cell membranes themselves. Alternatively, the enzyme may have a part to play, either in virus multiplication or in the release of newly formed virus from the cells, as will be mentioned later.

Viruses such as influenza may in fact be taken into cells by an active process of ingestion by the cells themselves. The process of pinocytosis, a property of macrophages, and also of the growing tips of nerve axons, as shown by the films made by A. F. W. Hughes at the Strangeways Laboratory, may be common to many types of cell, and provide a means of entry for many viruses.

Penetration of cells in *in vitro* systems need not take the same course as in the organized tissue of an intact organ. Here virus may spread from cell to cell directly, without passing through the fluid phase of the culture Vaccinia virus in cultures of chick fibroblasts (Noyes, 1953), or herpes B virus in cultures of monkey kidney or HeLa cells (Black and Melnick, 1955), form well-defined, progressively enlarging pocks, even when the cell layers are submerged in fluid without an overlay of agar to restrict diffusion of the newly formed virus. In both cases, however, virus is released into the fluid in addition to the cell-to-cell spread just mentioned, and at later stages new pocks can appear in the sheet of cells at a distance from the primary foci. A much more extreme case is the agent isolated by Weller (1953) from cases of varicella and herpes zoster, which, although it can be transferred from cell to cell and produces focal lesions in culture, can only be passed from culture to culture if infected cells as well as fluid are transferred.

Preliminary evidence has been obtained in many cases of the adsorption of other viruses to animal cells, but none have yet been analysed in detail. No evidence has yet been obtained, however, of an injection process akin to that found with phage. Animal viruses seem to find it easy to get into their host cells, and may be actively swallowed by them. Plant viruses seem to go to the other extreme. At their primary

sites of invasion they need to be placed inside their host cells before multiplication can proceed. In nature this is achieved by the mouth parts of a sucking insect or by a penetrating parasite such as the dodder; in the laboratory it is done with abrasives. Within the plant cell-to-cell spread is made easy by the existence of intercellular protoplasmic bridges.

Maturation and release of new virus

It has often been claimed that animal viruses like bacterio- [illegible] which the virus multip- [illegible] but non-infective form [illegible] However, it is only in the case of a small number of viruses which have been studied intensively in suspensions or monolayers of uniform cells in tissue culture that such a phase in the growth cycle has been rigorously demonstrated.

When bacteria are infected by the fully virulent type of bacteriophage, vegetative multiplication of the virus is followed by the accumulation within the infected bacteria of an increasing number of mature phage particles; when the number of these approximates to the 'burst size', the bacterium lyses and its content of virus is released. In the case of those animal virus growth cycles which have been studied in detail, it is difficult to separate the phase of replication from those of maturation and release of virus. Possibly because animal cells have much less rigid cell walls than bacteria, new virus seems able to escape from the cells almost as soon as it is formed. In the cases of influenza, Western equine encephalomyelitis and poliomyelitis, there is a period after the infecting virus has penetrated the cells during which no infectivity is recoverable, followed by a period during which new virus is released from the cells in an exponential manner. The time over which the leaking out takes place is very variable. In the case of Western equine encephalomyelitis virus growing in chick fibroblasts, the total yield per cell may be of the order of 1000 plaque-forming units. The release of new virus begins

about 2 hours after infection, and may go on for up to 12 hours. However, released virus appears in the supernatant fluid of the cultures at about the same rate as it does in the cells, and Rubin, Baluda, and Hotchin (1955) showed that of a total of between 100 and 1,000 particles produced by each cell, the maximum number of intracellular particles at any one time is only about 4–10 per cell. These estimates, however, refer only to the formation of mature virus, and we do not know how many vegetative replicating centres there are within the cell, and have no clue as to the manner in which they are set up. It does seem as though there may be a difference between phages and some animal viruses in the following sense:—the penetration of phage DNA into a bacterium initiates a series of duplications of the vegetative form; the new crop of phage particles then receive their extracellular equipment and are released in a group by the bursting of the cell. Cellular infection by an animal virus may cause the setting up of one or a number of centres for the manufacture of new virus particles, each of which can escape from the cell almost as soon as it is formed. Damage to the cells, however, does not appear to be correlated with the amount of new virus produced.

In the case of bacteriophage, the bursting of the infected cells at the end of the cycle of virus multiplication may be caused directly by the virus particles within them In this respect it is perhaps significant that the number of phage particles per bacterium needed to cause "lysis from without" is of the same order of magnitude as the burst size. Cell destruction by animal viruses cannot generally be ascribed to an assault on the cell membrane by the new crop of virus particles. Lwoff, Dulbecco, Vogt, and Lwoff (1955) studied the release of poliomyelitis virus from single infected cells and found that once new virus was formed, it leaked rapidly from the infected cells; cell death, at about 4 hours later, occurred when virus production ceased. Moreover, in chorioallantoic membrane cells infected with influenza virus there is a latent period of 2–3 hours before new virus begins to appear; once

this has happened the infected cells continue to release virus at a nearly constant rate for up to 30 hours or longer before the virus yield begins to decrease; necrosis of the infected cells does not appear until about 48 hours after infection. The escape of virus particles from infected cells must therefore cause only trivial damage to the cell surface, since cell integrity can be maintained for so long after virus production has begun. Additional evidence that the cell membrane is not disrupted comes from the fact that cell respiration continues unaffected throughout the period of virus release. The release of influenza virus from infected cells is accompanied by the extrusion of filaments from the cell surface. These have been studied both under dark-ground illumination and with the electron microscope. They will be discussed by Dr. Bang later in this symposium, and I will not deal with them further, except to point out that if filamentous extrusion is the sole method of virus liberation, then the cell may well resemble a self-sealing tank. Only when the virus productive capacity of the cell is exhausted (possibly by depletion of some key nutrient essential for both cell and virus) does cytopathic alteration become apparent. Schlesinger (1953) has described the existence of an intracellular substrate for the virus mucinase, which he has shown to decrease in amount during virus multiplication. However, the location of this substance within the cells is not known, and there is as yet no direct evidence that its destruction is related to the process of virus replication and not to the release of new-formed virus from the cells. There is some evidence that the mucinolytic enzyme is involved in the release of virus from the infected cells, since addition of the inhibitor AMPS to cells one hour after infection does not prevent virus production, though it does delay the release of virus from the cells. The latter process can then be greatly facilitated by treating the cells with RDE. If Schlesinger's mucinase substrate is situated close below the cell surface, it could well be the substance involved.

The hypothesis that metabolic derangement, perhaps involving depletion of some key nutrient, may be involved in

the cytopathic alterations which follow the infection of cells by some viruses is supported by further evidence from tissue culture work. Eagle (1955) has drawn attention to the similar- [illegible]

in both glucose and glutamine fail to support the propagation of polio virus. None of the other single or combined nutritional deficiencies which he tested had this effect. HeLa cells also respond to infection by the APC viruses by greatly increased acid production and the appearance of cytopathic change; there the addition of excess glucose will reverse the cytopathic effect, although it does not prevent the production of virus. Conversely, it is sometimes possible to interfere with the production of virus without influencing the development of the cytopathic effect. Ackermann, Rabson, and Kurtz (1954) studied the propagation of Type III poliomyelitis virus in HeLa cell cultures under conditions of massive infection. The growth cycle had a latent period of 4–5 hours, followed by a release of virus lasting 6–7 hours. Changes in the staining character of the cells appeared before the major part of the virus yield was obtained. The multiplication of both the cells and the virus is blocked by the addition of fluorophenylalanine, which stops virus growth at an early stage in the infection It does not, however, prevent the appearance of the cytopathic effect which goes on to develop at the ordinary rate.

Finally, abortive cycles of virus multiplication which do not result in the production and release of infective virus may, nevertheless, induce cytopathic change. Henle, Girardi and Henle (1955) have described such a case in the association of influenza virus and HeLa cells. Here definite specific cyto- [illegible]

cells.

Since these facts indicate that virus production and the

induction of cytopathic change can be dissociated by experimental means, it is perhaps a truism to point out that virus production can occur normally in cells without any obvious morphological change. A striking example is the Rous sarcoma virus, where infection of a cell by the virus initiates tumour development on the part of the cell, and the cell-virus relations in this respect have recently been determined in an elegant series of experiments by Rubin (1956). It seems that one tumour virus particle is adequate to initiate tumour development by the cell it infects, but that the release of virus *from infected cells is very low. A sarcoma cell suspension only* releases about one virus particle per 100 cells per hour. However, we do not know the extent to which even this relationship is determined by the environment of the infected cells, and whether the virus could not be made to destroy the cells in appropriate circumstances. A striking example of the effect on a virus-cell relationship of altering the environment of the infected cells has been given by Kaplan (1955) with poliomyelitis virus and monkey kidney cells. When monkey kidneys are injected *in vivo* with poliomyelitis virus, no cytopathic changes can be found and infective virus cannot be recovered either from perfused kidneys or cellular homogenates. However, if such kidneys are reduced to cell suspensions by treatment with dilute trypsin and the cells plated out as tissue cultures, cellular degeneration eventually occurs, with the release of new infective virus. *In vivo*, therefore, the virus is somehow maintained in association with the cells—even in animals with circulating antibody against the virus; when the cells are removed to an alien environment, the characteristic cycle of virus multiplication and cytopathic change makes its appearance.

Association of virus multiplication with cell organelles

Bacteria contain both DNA and RNA in large concentrations, but because of their sma ize we do not know very much about the way in which these are distributed within the

cells. The primary assault of a virulent phage is, however, an assault on the bacterial nucleus. The DNA of the bacteria is rapidly dispersed and replaced by that of the invading phage so that the genetic material of the phage rapidly takes over the entire cell. Animal cells, again, contain both DNA and RNA, but distributed in a better-known manner. The cell nucleus contains both DNA and RNA, the latter mainly concentrated in the nucleolus. In the cytoplasm there are the mitochondria, which are rod-shaped or spheroid and which contain undoubtedly a lot of phospholipid and protein, and possibly a little RNA. In addition there is the endoplasmic reticulum which is rich in RNA, and probably corresponds in the intact cell to the [illegible] which can be centrifuged out

plentiful in tumour than in other cells.

An abundant field exists for the investigation of the association between animal viruses in their intracellular phase and these well-defined cell organelles. So far, only scattered information is available. Herpes virus, for example, appears to multiply within the nuclei of infected cells. In herpes-infected chick embryo liver, a large portion of the virus, at any one time, seems to be associated with the nuclear fraction, though the percentage of the total virus so associated varies with the time after inoculation At the end of the cycle, that is about 12 hours after new virus has appeared in cultured cells, there is only a negligible amount of virus to be found associated with the nuclei in cell homogenates. But at the very beginning of the cycle, a very high proportion of the virus is found associated with the nuclei. Also the penetration of herpes into cells must alter the virus particles in such a way that they acquire an affinity for nuclei, since extracellular virus—that is 'mature' herpes virus particles do not selectively adsorb to isolated nuclei. Passage of virus through the nuclear membrane on the way out of the cells also may alter the particle structure. Electron microscope studies of infected cells by Morgan *et al.* (1954) showed that a limiting

membrane is acquired by the particles as they pass out of the nucleus into the cytoplasm, and that the particles there resemble mature virus. Reissig and Melnick (1955), who have studied the related B virus grown in monkey kidney cells, made a quantitative study which showed that new virus is found in the medium at the time when these completed double membrane particles can be found on the external *surfaces of the cells.*

Apart from a study by Friedlander *et al.* (1955), in which an association was found between Anopheles A virus and the endoplasmic reticulum, studies of this kind on other animal viruses have yet to be made. A profitable field appears to exist for correlation between quantitative virus studies and histochemical investigation of the infected cells.

Many of the cellular organelles mentioned above are themselves capable of replication. Under normal conditions of cell life, the multiplication of each particular kind of element must be matched with that of the others in the same cell. When an autonomous self-duplicating element, such as a virus, is introduced into such a system, we must expect the resulting incoordination to have a pathological result. The only exception will be where the multiplication of the intruder can be matched with that of similar elements in the cell; and the result will be to produce an intracellular symbiont whose presence will be undetectable.

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[Discussion of this paper was postponed until after the paper by Mr. Bawden.—Ed.]

membrane is acquired by the particles as they pass out of the nucleus into the cytoplasm, and that the particles there resemble mature virus. Reissig and Melnick (1955), who have studied the related B virus grown in monkey kidney cells, made a quantitative study which showed that new virus is found in the medium at the time when these completed double membrane particles can be found on the external surfaces of the cells.

Apart from a study by Friedlander *et al.* (1955), in which an association was found between Anopheles A virus and the endoplasmic reticulum, studies of this kind on other animal viruses have yet to be made. A profitable field appears to exist for correlation between quantitative virus studies and histochemical investigation of the infected cells.

Many of the cellular organelles mentioned above are themselves capable of replication. Under normal conditions of cell life, the multiplication of each particular kind of element must be matched with that of the others in the same cell. When an autonomous self-duplicating element, such as a virus, is introduced into such a system, we must expect the resulting incoordination to have a pathological result. The only exception will be where the multiplication of the intruder can be matched with that of similar elements in the cell; and the result will be to produce an intracellular symbiont whose presence will be undetectable.

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whether or not the host shows any adverse effects. Also, it is relevant to note that conditions that make healthy plants most likely to contract infection are not necessarily also those that favour the multiplication and accumulation of viruses in infected cells. This apparent paradox is, perhaps, most strikingly shown by keeping plants at different temperatures before and after they are inoculated (Kassanis, 1952). When plants are kept at 36° for a day or two before they are inoculated, their susceptibility to all viruses yet tested is much increased, and the same inoculum will produce many times more lesions than in plants kept at 15–20°. When plants are kept at the higher temperatures, after they are inoculated, far from the number of lesions being increased, most inocula produce fewer than at the lower temperatures and some viruses produce none. A comparable effect to the failure of viruses to multiply and cause lesions in inoculated leaves at 36°, and presumably with a similar cause, is the curing of plants systemically infected with some viruses by keeping them a week or two continuously at high temperatures At intermediate temperatures there is evidence that such viruses are being simultaneously synthesized and degraded and, in cells not killed by infection, viruses seem to resemble the normal cell proteins, at least in as much as that their concentration at any given time represents a balance between synthesis and degradation (Harrison, 1956a).

The reason for virus particles failing to multiply in plants at 36° cannot be decided with certainty. Prolonged exposure to [illegible]

It is, perhaps, more probable that the metabolism of cells at 36° favours degradation instead of synthesis of virus particles. That the physiological state of the host cell can alone determine whether or not introduced virus particles multiply is shown unequivocally when leaves are exposed to ultraviolet radiation before they are inoculated After suitable irradiation, leaves immediately resist infection by viruses to which they

THE MULTIPLICATION OF PLANT VIRUSES

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THERE are now many experimental results to show that varying the physiological condition of plants, by varying their environment or nutrition, greatly affects both the susceptibility of leaves to infection by manually inoculated viruses and the extent to which these viruses increase in infected leaves, but it is much easier to establish such phenomena than to explain them, for the processes involved in virus multiplication are largely unknown. To tell any consecutive story of the events between infection and the occurrence of newly formed virus still necessitates much speculation and argument by analogies with bacteriophages, and even then the story is far from complete.

As all the plant viruses yet studied are nucleoproteins, I shall assume that their synthesis does not differ fundamentally from that of other proteins. There is no evidence that any has any independent synthetic mechanisms or that virus particles change in size as might be expected if they multiplied by binary fission. On present knowledge, virus multiplication seems most profitably considered as an aberrant form of nucleoprotein metabolism (Bawden and Pirie, 1953), a process in which the whole cell is the synthetic system, with the virus one among many factors that determine the direction of its metabolism and its ultimate constitution.

There is no need here to describe the many and striking ways in which changes in the condition of plants affect their reactions to viruses, but it is worth stressing that the state of the host cells can determine whether or not introduced particles will become established and multiply and, if they do,

times after they are inoculated, three changes in the state of the particles can be detected and approximately timed. In the first state, the ability of the particles to multiply is unaffected by light; at 20° a few particles move from this state within 15 min, many do so within 30 min. and most within 1 hr. They can remain in the second state for 1–2 hr., but if within this time the inoculated leaf is not exposed to light, they move into their third state and are no longer susceptible to photoreactivation. The change from the first to the second state is reasonably interpreted as the time taken for the virus particles to enter the host cells or, equally likely, some specific cell component. By analogy with bacteriophages, this step may entail the nucleic acid separating from the protein; there is no direct evidence that infection with any plant virus

X can be photoreactivated, is approximately the same as that during which normal virus can be prevented from multiplying by treating inoculated leaves with pancreatic ribonuclease. The fate of particles that are not photoreactivated is unknown, but it is reasonable to assume that the nucleases and proteases of the host cells destroy them.

When leaves are inoculated, washed to remove unattached virus, macerated at various intervals, and the extracts assayed for their virus content, it is a simple matter to determine the rate at which virus increases in the leaves Provided conditions are kept constant, reasonably reproducible results are obtained with any given inoculum and host. After an interval ("latent period"), which depends on the temperature and the virus content of the inoculum, new virus becomes detectable, and the infectivity of successive extracts then usually increases steadily. The infectivity of successive extracts made during the latent period, however, usually decreases. Increasing temperature up to an optimum, which differs for different viruses, shortens the latent period and increases the rate at which new virus accumulates, but this

are normally susceptible, but provided such irradiated leaves are exposed to visible light, not only do they later regain their original susceptibility, but they may become even more susceptible than unirradiated leaves (Bawden and Kleczkowski, 1952; Benda, 1955). During their resistant phase, host cells seem able to inactivate virus particles that normally would have multiplied, for virus inoculated to leaves in this phase does not remain latent through it and then multiply when susceptibility is regained. The fact that cells are again susceptible becomes obvious only by inoculating with more virus. And this is so despite the fact that active virus can be recovered from such leaves. Virus apparently becomes established and multiplies only if it achieves some favourable condition soon after leaves are inoculated; if it does not, it either remains attached to the leaves but ineffective, or is inactivated.

The importance of the host cell's behaviour in determining whether inoculated particles remain apparently inert, or behave like viruses and multiply, is shown equally vividly by the phenomenon of photoreactivation. Except for tobacco mosaic, all the plant viruses as yet tested, when exposed appropriately to ultraviolet radiation, have given preparations that are more infective when the inoculated plants are kept in light than when they are kept in the dark. Whether illuminated cells repair the damaged particles, or whether conditions in illuminated cells so favour the first steps in virus multiplication that even damaged particles can take them, is uncertain. Whatever the explanation, the ability of such particles to multiply unquestionably depends on some light-sensitive mechanism provided by the host. From experiments with potato virus X, which shows the phenomenon of photoreactivation more strongly than other plant viruses yet tested, it is also clear that, for the mechanism to be effective, the damaged particles must encounter it soon after they are

Stronger evidence that the first cycle of virus formation is undetected is obtained by exposing inoculated leaves to ultraviolet radiation at different times after inoculation. Moderate exposures can be expected to inactivate virus in the epidermis but not in the mesophyll. Thus, by finding what interval between inoculation and irradiation decreases the number of lesions by half, the mean time can be found for virus to reach the mesophyll, which is presumably the mean time effective inoculum takes to multiply in the epidermal cells and for virus formed in them to spread to the mesophyll. [illegible] lesions to become visible in *N. glutinosa* inoculated with tobacco mosaic virus

Various reasons can be advanced to explain the failure to detect the virus first produced in the inoculated cells. It may be in some way immature, able to move to and infect neighbouring cells, but not yet stable enough *in vitro* to make an effective inoculum. Or the virus first produced in these cells may all be specifically and rapidly absorbed by components of nearby cells, so that it never becomes free in extracts. The simplest explanation, however, is that the first crop of virus from the epidermal cells infected at inoculation is too small to be detected by current inoculation methods, indeed too small even to offset the inactivation of ineffective inoculum. As already said, about a million virus particles are needed to get an infection by our methods of inoculation, and this is the order of size of the number of particles estimated to occur in fully infected cells (Nixon, 1956; Harrison, 1956*b*); there is, then, no call to wonder that the first formed virus passes undetected, particularly as tests are perforce made on leaf extracts containing substances that can inhibit infection. The amount, though, seems adequate to infect 100 or more nearby cells, and when these in turn have gone through the cycle of events that leads to new virus, multiplication becomes evident. The localization of infection by tobacco mosaic virus in *N. glutinosa*

optimum is usually above the temperature at which the maximum virus content of leaves is obtained. When the host reacts with a sharply localized necrotic lesion, virus usually increases only during the period when the lesions are also developing, but otherwise the virus content of the leaves continues to increase for many days, reaching the maximum slowly at low temperatures (Harrison, 1956*b*).

It is easier to get these results than it is to interpret them. We are dealing with a multicellular system of which only a small part of the surface becomes infected at the time of inoculation. Also, the fate of the inoculum is uncertain; most virus particles that remain attached to washed leaves seem not to infect, for under usual conditions about a million are retained for each infection. This type of experiment, then, cannot alone tell whether the fall in recoverable virus during the latent period has any significance in the course of events that leads to virus multiplication; or whether the first rise in the infectivity of extracts identifies the first crop, or generation, of new virus; or for how long the virus content of single cells continues to increase, and whether the period of greatest increase represents increasing numbers of cells becoming infected or virus increasing per cell.

From experiments with tobacco mosaic virus in *Nicotiana glutinosa*, it seems likely that the drop in recoverable virus during the latent period is irrelevant to the process of infection and, *indeed, that the amount recoverable from inoculated* leaves continues to fall even after new virus has been produced. The infectivity of extracts does not begin to rise until just before the necrotic lesions become visible, when many mesophyll cells are already infected and dying. As there is good reason to assume that only epidermal cells become infected at inoculation, it seems that the first generation of new virus is not detected in extracts, *for the* inoculated virus must presumably have multiplied in the epidermis, from which its progeny spreads to and multiplies in the mesophyll, before the virus content of extracts increases detectably.

tobacco mosaic virus appears in quantity in infected leaves, the amount of insoluble nucleic acid increases by an amount that is slightly in excess of the amount ultimately appearing in the virus and it decreases again as the virus increases. This rise and fall of insoluble nucleic acid become detectable only some days after leaves are inoculated, long after irradiation ceases to prevent lesion formation, but this difference in timing does not mean that the phenomena are not related. Experiments with ultraviolet radiation detect effects only in the epidermal cells infected at inoculation, whereas the chemical tests detect effects only when virus has begun to multiply in a large proportion of the leaf cells, probably when the third cycle of virus multiplication is initiated in a leaf.

As the virus content of infected leaves increases, so too it becomes possible to show that virus multiplication is not the replication of an infecting particle to give a uniform, stable product. Since Bawden and Pirie (1945*a*, *b*) found that plants infected with either the Rothamsted tobacco necrosis virus or tobacco mosaic virus contain more than one anomalous protein, the number of such proteins described in plants with tobacco mosaic has steadily increased (Takahashi and Ishii, 1952, 1953; Commoner, Newmark and Rodenberg, 1952; Jeener and Lemoine, 1953), and another particularly striking example found in infections with turnip yellow mosaic virus (Markham and Smith, 1949). These different proteins have been identifiable because they differ from one another either in size, weight or electrophoretic mobility, and when separated from one another they have been found to differ in their infectivity and chemical constitution. It has been generally assumed that the various proteins produced by infection with a given virus strain are antigenically similar, but this may not be true. That some contain antigens not present in others is strongly suggested by the fact that more than one line is obtained when precipitation tests are made by the gel-diffusion method (Jeener, Lemoine and Lavand'Homme, 1954; van Slogteren, 1955, Kleczkowski, 1956), and the similarities previously suggested may mean that the antisera

also has a ready explanation on the basis of this process: if only mesophyll and not the epidermal cells are killed by the multiplying virus, then virus synthesized in epidermal cells infected at inoculation will be able to spread to the nearby mesophyll, which, however, will die before the virus produced in it can spread to a third lot of cells.

Exposing inoculated leaves to different amounts of ultraviolet radiation also suggests changes in the state of the virus in the initially infected epidermal cells, though it identifies none as early as does the phenomenon of photoreactivation. An hour after inoculation, when particles have become susceptible to photoreactivation, the virus is still as readily inactivated by ultraviolet radiation as immediately after inoculation, but after another hour they are much more resistant and their resistance continues to increase until the time when irradiation no longer prevents lesions from developing (Bawden and Harrison, 1955). With Rothamsted tobacco necrosis virus in French bean there was only slight indication that the inactivation curves changed from being straight to curved at about two-thirds of the time needed for irradiation to become ineffective, but with tobacco mosaic virus in *N. glutinosa* the published curves (Siegel and Wildman, 1956) clearly suggest that the cells contain more than one infective, or potentially infective, particle at this time. The increased resistance to inactivation at 2 hours after inoculation is reasonably interpreted by assuming that the infected cells have synthesized
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eaf is
detectably increased (Owen, 1955).

The suggestion that the increased resistance to inactivation by ultraviolet radiation results from infection changing the nucleic acid composition of cells, receives some support from chemical studies comparing the nucleic acid of healthy and infected leaves. Basler & Commoner (1956) report that, before

bean form of the inoculum multiplies extensively without the leaves showing lesions.

Much more of the specific nucleoprotein produced in bean than in infected tobacco seems to be non-infective, and to get similar numbers of lesions in *N. glutinosa* inoculum from beans needs to be more than ten times as concentrated as inoculum from tobacco. The differences between the properties of the main nucleoprotein formed in bean and tobacco suggest that the protein parts of the particles from the two hosts differ considerably. After a long time in either of these hosts, during which the virus has passed through a series of plants, it will still return to the other, with its properties there apparently unchanged. Something, then, seems to be stable and to give genetical continuity; on current views this something is most plausibly regarded as the nucleic acid, and the occurrence of this strain in its various guises is perhaps evidence of the range of proteins with which the same nucleic acid can be successfully allied. If this is so, and there is every reason to think that the nucleic acid would encounter widely different proteins in cells with such diverse metabolisms as bean and tobacco, questions about the functions of the protein almost suggest themselves. Does its structure determine the ability of particles to move from cell to cell inside the leaf? And the type of symptoms caused? These questions cannot yet be answered, but inoculations with this strain clearly show that plants continue to produce virus that resembles the inoculum for as long as this type can spread from cell to cell without killing them, but in conditions where it can not, it becomes superseded by another form that can.

The main interest of this unusual strain of tobacco mosaic virus is, perhaps, that it makes more obvious than previously that virus multiplication is not simply the replication of the initially introduced particles and that the constitution of the final products can differ greatly from that of the inoculum. Thus, although some part of the characteristic particle of tobacco mosaic viruses may be self-replicating, as with the tadpole-shaped bacteriophages, much of it seems not to be.

used were prepared against mixtures of proteins not all antigenically identical. It is probably safe to assume that we are still far from identifying all the anomalous proteins that occur in infected plants, and of their biological significance we know little or nothing From work with radioactive isotopes, van Rysselberge and Jeener (1955) suggest that the small, non-infective, particles in plants infected with tobacco mosaic virus are precursors of larger particles, but as the ratio of small to large particles can be changed in fully infected leaves by altering the conditions under which they are kept (Bawden and Pirie, 1956), it seems, too, they can be produced by the degradation of larger particles. Also, there is no reason to conclude that all specific particles that are non-infective are necessarily precursors or breakdown products of larger particles; some may well be concomitant products of protein synthesis that were never destined to be incorporated in infective particles. Certainly, the ratio of small to large particles differs characteristically in infections with different strains of tobacco mosaic virus, and it can sometimes be altered by changing the conditions in which infected plants are maintained.

The ability of the host plant, and of the state of the host plant, to determine the products of virus multiplication is strikingly shown by a strain of tobacco mosaic virus that infects French bean systemically (Bawden, 1956). As obtained from systemically infected tobacco leaves, this strain closely resembles ordinary tobacco mosaic virus. As obtained from systemically infected bean leaves, however, it has widely different physical properties, antigenic constitution and causes different symptoms in many plants; it then causes local lesions in both tobacco and *N. glutinosa* at 20° and the local lesions in *N. glutinosa* take twice as long to appear and are less than half the size of those formed by the virus obtained from tobacco. At this temperature young tobacco plants inoculated with the bean form also produced the alternative form of infective particle, which then becomes systemic. At 30°, however, the alternative form is not produced, but the

synthesis and degradation may balance one another. The maximum with one virus and one host can be varied by altering the condition of the host, but the variation is small by comparison with the difference between the maximum achieved by different viruses. It is perhaps significant that only viruses that are unusually stable *in vitro* reach high concentrations *in vivo*, and it is worth comment that a stable virus like tobacco mosaic virus can be the main protein of infected tobacco plants.

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Our incomplete story can be briefly summarized: the virus content of leaves continues to increase for several days after they are inoculated with a virus that becomes systemic because an increasing number of cells become infected during this time, by virus spreading from previously infected cells where it has already multiplied. In each cell, it is reasonable to assume that the sequence of events in the inoculated epidermis is repeated, but with the ultimate result more certain, for if virus from the inoculum is inactivated in the epidermal cells it is not replenished, whereas the supply of virus for the cells invaded later will be almost unlimited. At each new invasion, the infecting particles can be expected to change their state, either as a preliminary to entering the uninfected cell or, perhaps, a specific component of it. This change is soon followed by the appearance in the cells of new products, nucleic acid and proteins, probably produced separately and then later combined, first to form relatively small particles which later coalesce, or aggregate, to produce the typical rod-shaped particles. There is little novel in the idea that tobacco mosaic virus particles do not emerge fully formed in one step, but are built up from smaller units; although this view is only recently being canvassed with enthusiasm, it has been known for more than 10 years that the small quasi-spherical particles of anomalous protein in extracts of infected plants can be changed *in vitro* to rods, morphologically indistinguishable from those usually considered typical of virus particles (Bawden and Pirie, 1945*b*). This change, though, does not make them infective, and to acquire this property the units may need to be built together in specific arrangements. Not all the long rods put together *in vivo* are infective, and of those that are, not all precisely duplicate the original infecting particles. The main character necessary for a particle to survive in a cell is probably that it should be stable there, rather than it should possess any specific biological activity.

In each infected cell, virus synthesis probably starts within a few hours and then, unless the cell is killed, the virus content increases for a day or two, to reach a maximum at which

reversible, as Wagner has shown on some of the influenza work on tumour cells recently (Wagner, R. R. (1955) *Proc. Soc. exp. Biol., N.Y.*, 89, 103) This virus is not deep in the cell Then the membrane which is continuously changing and engulfing fluid droplets takes in whatever is stuck on the surface and that goes down into the cell and is no longer released by RDE.

Now, I don't think that pinocytosis is the answer to the basic question of how the virus initiates the combination with the cytoplasmic components so that it can start to multiply. It merely is a disturbing phenomenon in terms of trying to understand what is absorbed and what is not absorbed and may obscure the basic issue as to how the virus joins the host cytoplasm. But it is such a common phenomenon in many cells that it undoubtedly changes the figures on absorption in various ways

Watson: Mr. Bawden, have you considered using fluorescent anti-

[illegible]

as 4 or 5 hours

Bawden Yes.

[illegible]

[illegible] Mr. Bawden, was the inhibition of multiplication by rubbing over the leaf obtained using pancreatic ribonuclease?

Bawden: Yes.

Ada Have you tried similar experiments with Pirie's leaf ribonuclease?

Bawden: No, I think it is almost certainly a much less powerful inhibitor, but we have never been able to make critical comparisons

DISCUSSION

Bang: I should like to ask Dr. Sanders a point on, perhaps, internal consistency. How is it that you picture a cell as having a dynamic membrane and at the same time talk about a limited number of receptor units?

Sanders: I really meant that at any instant there was a limited number of spots at which virus can get in or out. Is that inconsistent

number of spots in such a system

Sanders: You are conceiving of the spots as being re-synthesized?

Bang. Yes, I think there is good evidence for this.

Sanders: I was not thinking of the membrane as being a static structure; merely that at any *one* time there are a number of places

necessary.

Western equine encephalo-
mbryo cells in culture, the
ate calculation of the effic-
hat this efficiency is of the
to be relatively very high.
ytosis in view of these two
facts?

Bang: I think that I have been guilty in the past of some confusion

reversible, as Wagner has shown on some of the influenza work on

phenomenon in terms of trying to understand what is absorbed and what is not absorbed and may obscure the basic issue as to how the virus joins the host cytoplasm. But it is such a common phenomenon in many cells that it undoubtedly changes the figures on absorption in various ways.

as 4 or 5 hours.

Bawden: Yes.

Lwoff: Dr. Manigault, in the Pasteur Institute, has injected *Pseudomonas tumefaciens* into plants and could demonstrate very easily the antigens on sections with Dr Coons's technique. So this technique is

over the leaf obtained using pancreatic ribonuclease?

Bawden: Yes

Ada: Have you tried similar experiments with Pirie's leaf ribonuclease?

Bawden: No. I think it is almost certainly a much less powerful inhibitor, but we have never been able to make critical comparisons

because the leaf ribonuclease has not been obtained in the same state of purity as the pancreatic ribonuclease.

Ada The question of the relation between the soluble antigen of the tissue and of the virus particle that we were discussing this morning

Schäfer · Is there some evidence in your case, too, that the different lines you observed are not due to different size of the same antigenic material? I ask this because we found—as *reported*—that our preparations of purified s-antigen from fowl plague contained particles of different size

Ada · Becker has done some work on that aspect. I don't think his

kept at 36°?

Bawden Keeping healthy plants at 36° instead of, say, 20° before they are inoculated increases their susceptibility to infection. This

samples produce only small lesions but later samples produce large

very insignificant multiplication of the virus and such virus gives very irregular titration results in eggs, whereas the same virus, incubated at 33°, will produce death of the eggs with the development of very much more virus.

We hope that tissue culture methods will help to sort out this problem in the case of blue tongue. Blue tongue virus titrated in tissue culture by the Dulbecco plate technique has in some of our experiments produced with concentrated inoculum no plaques at all, but higher dilutions ... produced plaques. Virus recovered from such plaques has
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...rhaps containing DNA,

and

(3) "networks", which were acidophil concentrations in the cytoplasm looking like bits of wire netting, and which may perhaps correspond to what are called "matrices", as shown in electron micrographs by Gaylord and Melnick.

If you take the percentage of infected cells showing the first type of inclusions—the elementary bodies—and plot them against time, you get a very rapid fall in about 1 5 hours, after 10 hours there is an increase So that elementary bodies are present at the beginning

within the cells, disappear very rapidly and reappear in about 10 hours (Figure 1).

If you take the second kind of inclusion—the homogeneous bodies—many are present at the beginning, but this number falls very fast and they never reappear again (Figure 2).

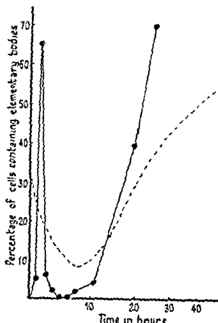
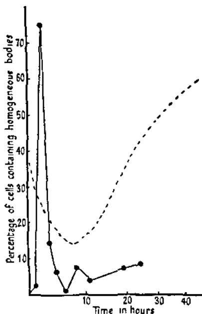


FIG. 1 (Sanders). Comparison of the percentage of cells containing vaccinia elementary bodies at various times after infection, with the growth curve of vaccinia virus in skin maintained *in vitro*. Solid line, percentage of cells containing elementary bodies (from data of Bland and Robinow); dotted line, vaccinia growth curve (present data).

Figs. 1–3 reproduced by courtesy Quarterly Journal of Microscopical Science

And the third type—the "networks"—you find there are few at the beginning, but that they increase later in the manner of Figure 3. One could account for these observations by making a hypothesis that the virus gets into the cells as elementary bodies; the appearance of the

homogeneous bodies is then an index of some sort of transformation



within the cells, disappear very rapidly and reappear in about 10 hours (Figure 1).

If you take the second kind of inclusion—the homogeneous bodies—many are present at the beginning, but this number falls very fast and they never reappear again (Figure 2).

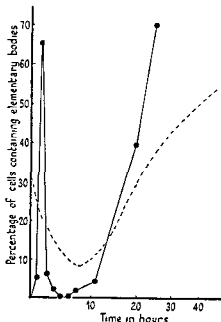


FIG. 1 (Sanders) Comparison of the percentage of cells containing vaccinia elementary bodies at various times after infection,

Figs. 1–3 reproduced by courtesy Quarterly Journal of Microscopical Science

to 20 to 25 per cent during the so-called eclipse phase. We have not published these observations.

particle is not partially blocked by various cell constituents, cytoplasmic constituents, and so on. I was wondering whether anybody had ever studied small bacteria like *Tularense* or even *Abortus*, by titration methods in the chorioallantois to see whether these small

have been collaborating with Dr. Valentine at Hampstead. We have

rabbit skin at 2, 3 and 4 days, and the ratio of total infected particles

an apparent eclipse phase.

Anderson's work (Anderson, S. G. (1954). *Aust. J. exp. Biol.*, 43, 633) with vaccinia was done on exactly the same lines as Sigel's (Sigel, Girardi and Allen (1951), *loc. cit.*) and I thought it provided a valid

to be highly Feulgen-positive with no Feulgen-positive area around them I think this is reasonably good evidence that APC's are probably DNA viruses.

Downie: With regard to this question of a latent phase or eclipse phase in pox viruses, we have done a good many experiments in the

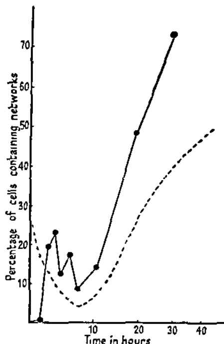


Fig. 9 (Sanders). Comparison of the per

data)

STUDIES ON MIXED INFECTIONS WITH INFLUENZA VIRUSES*

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SYSTEMATIC studies on mixed virus infections have been carried out in our laboratory over the past five years and in this paper we will attempt to bring together some of the more significant aspects of this work. Two brief new experiments will be described but the discussion of former work will be very compressed and the reader will have to consult the original texts for a more comprehensive account. Burnet and his collaborators have published extensive evidence since 1951 on a number of pairs of influenza strains, showing that recombination occurred under various conditions of mixed infection. This work has been very adequately reviewed (Burnet, 1955) quite recently and no systematic attempt will be made to refer to it in this account.

In order to understand the limitations of this type of investigation it is necessary to describe briefly the techniques which were employed. The mixed infections were usually carried out in the allantoic cells of intact or de-embryonated eggs. The virus input was large, usually more than ten infective particles per cell, and the yield was taken from the virus shed into the allantoic fluid The limiting infectious dilution method was used to isolate pure clones from mixtures. Infective material was inoculated into eggs in such dilution that 25 per cent or less would become infected. There is some evidence that most eggs are infected by single particles under such circumstances (Liu and Henle, 1953; Gotlieb and Hirst, 1954). This method

* These investigations were supported in part by research grants E-377 and E-675 from the National Institute of Allergy and Infectious Diseases of the National Institutes of Health, Public Health Service.

that is to say, virus can be recovered from leaves soon after they have been inoculated, but not some hours later. When more concentrated inoculum is used, virus can be recovered all the time, but more is recovered soon after inoculation than some hours later. This decrease in recoverable virus may well be irrelevant to the process of infection and simply reflect the inactivation of surplus inoculum. The decrease continues for about twice the time we think necessary for virus in the inoculum to multiply in the initially infected cells and pass from there to deeper cells This we think we can time by exposing inoculated

leaf extracts. Apparently the cells initially infected produce enough

virus that produces large lesions. But the virus that produces these large lesions is not obtained from tobacco immediately newly formed virus becomes detectable; for some days the virus produces only the small lesions. And whether the

tobacco plants or in plants at 36°. That the purified preparations con-

possible.

coiling occurs and hence its later application over the genetic core would be understandably somewhat non-specific Virus particles are capable of accepting coat material from rather distantly related agents (NDV and influenza A, for example) (Granoff and Hirst, 1954), but the mutual exchange is greater with closely related viruses such as two influenza A strains.

[illegible]

Morgan (unpublished) have electron photomicrographs of influenza infected allantoic cells in which fully capsulated particles with internal structure can be seen in beautiful array just outside the cell membrane Just beneath the cell surface are similar particles in a stage of partial capsulation. It does not seem too wild an inference to say that some early stages of virus multiplication, such as replication of RNA, takes place in the nucleus, and that coating of particles occurs as new material moves to the cell periphery. Maturation and extrusion seem to be almost simultaneous events These findings fit in very well with the current concept of phenotypic mixing.

Most of the subsequent studies to be described were carried out with two A strains of influenza, WSN and Melbourne The former is mouse virulent (IC) and the latter is avirulent by this route. The two strains will be designated as W+ and M −. They are antigenically related but still quite different from each other, and adsorbed sera were available which were specific for each. In our early experiments with these two strains, the phenomenon of phenotypic mixing was found and, because we erroneously thought that this might have a genetic basis, attempts were made to isolate an antigenic recombinant which was stable Hence, a long series of passages were carried out with rather complicated results but it may be worthwhile briefly to review this effort together with some new material.

The basic procedure (Hirst and Gotlieb, 1953b) consisted of [illegible]

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is so cumbersome that for practical purposes it is impossible to use it directly for the isolation of rarely occurring recombinants. Specific immune sera were used for suppressing one or another entire class of particles in mixed populations, and in this way clones of rare particles were obtained. However, this necessitated the use of parent strains which differed from each other serologically and, incidentally, differed in a large and unknown number of other respects, and this can be a disadvantage in recombination experiments. Another difficulty with the method is that one cannot usually differentiate between exceptional, unstable forms and the rare occurrence of multiple infection in isolation by chance.

The phenomenon which we first encountered (Hirst and Gotlieb, 1953*a*) is called phenotypic mixing, due to the fact that the genotype and phenotype in individual particles do not match. and frequently the phenotype is a mixture of parent-type specificities. The details with bacteriophage were carefully worked out by Streisinger (1954, 1956) and with influenza virus are most readily understood from consideration of studies on mixed influenza A-B infection (Gotlieb and Hirst, 1954), since there is no complicating genetic reaction between these two strains. A and B strains are antigenically completely distinct in both the haemagglutination inhibition and neutralization tests, and it was surprising to find that as many as 90 per cent of the particles from a mixed A-B infection were of mixed antigenic structure, and reacted with both antisera. Nevertheless, such particles were shown to be genotypically *either* A or B, but not both.

In trying to understand these results, it is helpful to picture the virus antigens as part of an outer coat surrounding a core of genetic material. One might imagine that in the cell the genetic core of a future virus particle would induce the formation of specific antigens on its own surface. However, the material might be tightly coiled in the final stages of

demonstration. A mixed M-W infection was carried out in the allantoic sac. The virus from this infection was titrated twelve times and fluids from all eggs were examined for the predominance of mixed M-W particles. Of course, phenotypic mixing was found in eggs that received low dilutions of virus, but in one titration mixed particles were found in an egg that had received a 10^{-5} dilution. Passage of this material produced more antigenic mixed particles in high dilution, etc, and hence it was an X-2 form which appeared in initial passage

The same experiment was performed with M and W strains that were obtained as segregants from an X-2 form. Mixed infection was carried out in an egg and the yield was titrated twelve times. All twelve titrations contained eggs that had received dilute virus and which showed the X-2 form. The fact that the form was X-2 was confirmed by passage.

These results show that there may be a special pair of M and W [illegible]

heterozygote which is less stable appears in mixed M-W infections. This was shown in the following way.

From a study of mixed A-B infections an estimate was obtained of the frequency of two particle infection in terms of the number of ID_{50} being inoculated. Following this, the progeny of a mixed M-W infection were inoculated into eggs at dilutions where the probability of two particle infection should have been very small The yield from these eggs was tested to see how many yielded one and how many both serotypes. The number of double yielders was much greater than would have been expected by chance, leading to the

summary, it appears that certain lines of M and W virus have an affinity for one another which gives a very large proportion of serotypic heterozygotes on mixed infection and this affinity

were tested for the presence of virus of mixed (M-W) antigenic type. In general, eggs that had received 0·01 ml. or more of allantoic fluid did yield virus in which antigenic mixing was predominant (provisionally called X-1 virus) and this continued throughout eight passages. Eggs that received less than 0·01 ml. of allantoic fluid yielded predominantly M virus, the fastest growing strain, and many eggs at terminal dilutions yielded W virus.

This may be explained as follows: when relatively equal *amounts of strains M and W were inoculated into an egg and* when the total inoculum was small, M virus predominated in the final yield since it grows more rapidly than W. An exception was at terminal dilutions where some eggs received W virus only. The great predominance of M prevented demonstration of phenotypic mixing. When large mixed M-W inocula were used the yield contained approximately equal numbers of the two genotypes and this balance persisted even through numerous passages, because large inocula prevented recycling and nullified the advantage of M's rapid growth rate. With equal numbers of the two types being produced phenotypic mixing was common and easy to detect.

In the 10th serial passage, a new phenomenon appeared (Hirst and Gotlieb, 1953*b*). Numerous eggs that had received high dilutions of passage virus produced virus which was predominantly of mixed antigenic type. This form (provisionally called X-2) was not the predominant particle in every egg but it could be readily propagated serially by passing dilutions of X-2 fluids. However, X-2 was unstable and continued to revert to parent types on passage. Evidence

heterozygous for several characters and may be doublets or diploids.

An experiment was carried out which shows that the X-2 form is potentially present in the progeny of every mixed M-W infection and that serial passage is not necessary for its

Methods for determining these markers were relatively simple. Detection of virulence in the mouse depended on the outcome of inoculating 0·03 ml. of a 10^{-1} dilution of allantoic fluid into mice by the intracerebral route. The details of this test have been fully described elsewhere (Hirst and Gotlieb, 1955). This marker was very good in the sense that most strains were either virulent or avirulent and very few equivocal results were obtained.

The presence of M antigens in W strains and W antigens in M strains was determined by neutralization tests in which the viruses were titrated in normal and in highly specific absorbed immune serum at a dilution of 1 16. In the case of W strains, each test was repeated five times. When the serum reduced the titre of the virus by 0·5 log. or more, the result was considered significant. With the serum used for these tests, reductions in titre up to 1·5 log. were observed With other sera, even more marked neutralization was noted. The sera were specific against the wild type strains

For absorption on aluminum phosphate (Miller and Schlesinger, 1955), one volume of allantoic fluid was added to one volume of 0 5 M phosphate buffer pH 7 4 Two ml. of this mixture was added to 2 mg of wet aluminum phosphate gel which had been previously equilibrated with 0 25 M phosphate buffer pH 6 3. After thorough mixing and standing for 30 minutes, the gel was removed by centrifugation and the supernatant together with unabsorbed virus were tested for HA titre by the pattern method. Adsorption was considered significant when 75 per cent or more of the HA was removed. When less than 50 per cent was removed, it was considered to be no absorption. Most tests fell sharply into one category or the other

Mixed infection of M− − − and W+ + + was carried out with large inocula in the allantoic sac The allantoic fluid from one egg was diluted out and 100 eggs inoculated of which 28 were found to be infected Of these, three yielded M virus alone, seven yielded W virus alone and 17 yielded a mixture of M and W. The virus from the single yielders was parent type

is strong enough that these forms can be maintained with little difficulty through serial passage. The wild types of M and W virus also produce heterozygotes but of lower affinity and these particles cannot be so readily propagated together *in series. The importance of these forms for recombination* will be reviewed below.

Recombination with Strains M − and W +

In our original effort (Hirst and Gotlieb, 1953*b*), we were attempting to get recombination between the antigenic components of strains M and W. After some 30-odd passages of the X-1 and X-2 form, a line of virus was isolated which was antigenically predominantly W, but also contained a definite otherwise specific M component. This was shown by the fact that this virus was strongly neutralized by type specific anti-W serum and to a lesser extent by type specific anti-M serum. The meaning of this finding was not entirely clear at that time, but it is now apparent that the new strain (provisionally called X-3) was essentially W virus which had acquired a small M component by recombination It has also been recently realized that serial passage *per se* had nothing to do with the appearance of this form.

The following small experiment is cited to show how recombination of antigens and two other markers occur following mixed infection with M and W virus. The two parent strains were characterized as follows: strain W was highly virulent for mice (V +) IC; it contained none of the specific antigens of strain M (ant. +); it was readily absorbed from allantoic fluid by aluminum phosphate at pH 6·3 in the presence of 0·25 M phosphate (abs. +). The M strain was completely avirulent for mice (V −); contained no W specific antigen (ant. −) and absorbed poorly on aluminum phosphate in the presence of 0·25 M phosphate buffer at pH 6·8. Thus, in relation to mouse virulence, the occurrence of heterogeneous antigen and absorbability on aluminum phosphate the W strain was + + + and the M strain was − − −,

between replicating genetic material from two particles that have entered the cell at random.

Some recent experiments have been carried out on reactivation of irradiated M− virus by active W+ (Gotlieb and Hirst, 1956). In one sense, these experiments merely confirm the earlier discovery by Burnet and Lind (1954) that heat-inactivated virus could be reactivated by active virus and the reactivated strains were recombinants. Our inactivation was done with ultraviolet light, and reactivated M− strains were frequently but not always virulent for mice (M+) by a back-cross test with W−. In this case, we have much better evidence than has been obtained heretofore that reactivation occurred secondary to recombination. In previous cases (Burnet and Lind, 1954; Baron and Jensen, 1955), the possibility was not eliminated that reactivation took place by some non-genetic mechanism and that recombination was secondary and incidental. These experiments point to the possibility of obtaining rare recombinants through the mechanism of reactivation and is especially applicable to those cases where recombinants are infrequent.

We had hoped through these studies to discover something about the genetic background of virulence. From the origin of the WSN strain, it seemed likely that virulence was polygenic in character. However, the readiness with which virulence was lost and was then fully restored by a number of M+ strains suggests that the number of factors involved in the variation from W+ to W− were either small or else they were closely linked.

It is clear that the advent of the plaque technique has rendered obsolete the approach which has been employed in the foregoing experiments. For several years, work has been under way in this laboratory studying NDV plaques on chick fibroblast monolayers (Granoff, 1955). NDV has many advantages for genetic work since it is relatively stable, has high plating efficiency, there is no von Magnus type of phenomenon, and markers involving haemagglutination are available. The results, thus far, do not justify a detailed presentation

(either M−−− or W+++) in every case. From the 17 double yielders, 17 M strains were tested and all were found to be M−−− like the parent strain. Of the 17 W strains, five were W+++ (parent type), seven were W−−−, two were W+−−, two were W++− and one was W−+−.

In a later experiment (Hirst and Gotlieb, 1955) with this same pair of strains and with a mouse virulent M strain (M+), an attempt was made to get some other quantitative results. An approximation of the findings from two crosses is as follows

$$\mathrm{M}- \times \mathrm{W}+ \;\rightarrow\; \begin{array}{l}\mathrm{M}-\\ \mathrm{W}+\\ \text{heterozygotes}\end{array} \begin{array}{l} \nearrow \left\{\begin{array}{l}\mathrm{M}-\\ \mathrm{W}+\end{array}\right. (50\ \%)\\ \searrow \left\{\begin{array}{l}\mathrm{M}-\\ \mathrm{W}-\end{array}\right. (50\ \%)\end{array}$$

$$\mathrm{M}+ \times \mathrm{W}- \;\rightarrow\; \begin{array}{l}\mathrm{M}+\\ \mathrm{W}-\\ \text{heterozygotes}\end{array} \rightarrow \left\{\begin{array}{l}\mathrm{M}+\\ \mathrm{W}+\end{array}\right. (100\%)$$

The common factors in these experiments were: (1) Recombination was found almost exclusively in the progeny of heterozygotes. (2) The recombination results were very asymmetric. Only one of the two parent serotypes appeared regularly as a recombinant. M virus appeared rarely as a recombinant. (3) The three characters tested showed some linkage, but in one-third of the recombinants less than three new characters appeared.

The importance of the heterozygote may well be something rather peculiar to this M-W pair, but this exceptional behaviour may nevertheless shed some light on mechanisms of virus multiplication. In considering the way in which influenza particles are coated before extrusion, it seems not impossible that two genetic units might accidentally be extruded in one coat, forming a doublet. Such a particle might have an excellent chance of producing recombinants, since the genetic apparatus of two particles would enter the cell at once in the same location. Certainly, the large volume of the host cell must be a considerable barrier to contact

[illegible]

Knight: Our cross-reaction, we feel, is due in part to incorporation of the same normal component in the virus particles.

Burnet: There is a point about the behaviour of M recombinants which might be of use. In the actual pair used by Dr Hirst, I think the NWS is different from mine?

[illegible]

methods Has that been tested, do you know?

[illegible]

Burnet: Where some types of virus are concerned, I think that is

and analysis at this time, but a short account may give an idea of the difficulties involved. The Beaudette strain of NDV gives mainly large plaques on plates and the wild type is relatively virulent for chick embryos. One in 200 particles of the wild type is a small plaque former and these small plaque formers are relatively avirulent in the chick embryo. Large plaques and virulence are not due to the same gene, since small plaques and virulence or large plaques and avirulence have been obtained together by recombination and other experiments. The problem which arises is why two changes in character are found simultaneously and it appears unlikely that it is due to mutation. An explanation of this phenomenon is required before the mechanisms involved in recombination can be understood.

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DISCUSSION

THE MORPHOLOGICAL ASPECTS OF VIRUS INFECTIONS OF CELLS AS REVEALED BY FLUORESCENT ANTIBODY

ALBERT H. COONS
Harvard Medical School, Boston

ANTIBODIES labelled with fluorescein have been successfully employed to detect the antigens of a number of viruses inside the infected cell: mumps, influenza, infectious canine hepatitis, vaccinia, varicella, primary atypical pneumonia, "Egypt", canine distemper, measles, herpes simplex, psittacosis, and, in preliminary experiments, poliomyelitis. It is likely, therefore, that cells infected with most viruses against which antibody can be obtained either by the immunization of animals, or from individuals convalescent from infection, can be visualized in this way.*

There are two ways in which labelled antibody can be used for this purpose. The first is the obvious one of labelling the specific antiserum. The second employs diluted unlabelled specific serum, which is allowed to react with the antigens in the cells. After the reaction, the unreacted portion is washed off, and the deposited globulin can then be found by means of fluorescein-labelled antiglobulin serum. This second method is sparing of specific serum and has a greater sensitivity, presumably by virtue of the favourable antibody-antigen ratio in the antibody excess range. It is also the only method available for the study of agents for which there is no satisfactory preparation of antigen for immunization, since it allows the use of convalescent serum the titre of which may be too low for direct use Varicella (Weller and Coons, 1954),

* The failures recorded in an earlier summary (Coons, 1951) have been made good in the cases of poliomyelitis Type II (Lansing) and Theiler's GD VII virus

very definitely so. With the M+'s, the intermediate ones, if you do

something different.

cells later.

minutes. We don't know how long it lasts, for the time being We have found the same effect for poliomyelitis virus.

Burnet: I don't think that can hold in the de-embryonated egg with influenza.

Dulbecco: I don't think it has been proved, because it seems to me that the only way to prove it is to take the cell suspension, and infect it and see what proportion of the cells release virus

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measles (Cohen *et al.*, 1955), primary atypical pneumonia (Liu and Eaton, 1955), psittacosis (Buckley, Whitney and Rapp, 1955), and herpes simplex (Lebrun, 1956) have all been visualized by this indirect method. The technical procedures employed have been discussed in a recent review (Coons, 1956).

The resolving power of a method employing fluorescent antibody and the fluorescence microscope is not such as to allow observations on the earliest stages of the infection of a cell. In the case of infection of the amnion of the developing chick embryo with mumps virus, the first detectable antigen appeared as tiny fluorescent granules in the cytoplasm, probably representing small "colonies" of virus particles (Watson, 1952). In the case of herpes simplex infection of human carcinoma cells in tissue culture, Lebrun (unpublished) found the first manifestation of the infection as small fluorescent spheres inside the nucleus, a finding consistent with the electron photomicrographs of the herpes-infected chorioallantois published by Morgan, Ellison, Rose and Moore (1954), which show aggregates of particles in the same location; it seems likely that the two results are comparable despite the difference in host cell. In this case, then, the sensitivity of fluorescent antibody is of the order of perhaps a few hundred particles in a closely packed aggregate.

The earliest stage of influenza infection in the amnion has a quite different appearance; viral antigen is first detectable outlining the cell-nucleus. The antigens of this agent subsequently fill the cytoplasm diffusely, both in the amnion of the chick (Watson and Coons, 1954) and in the nasal epithelium of the ferret (Liu, 1955). Its multiplication seems to begin in close association with the nuclear membrane.

Three viruses producing classical inclusion bodies have so far been investigated with some cytological precision by means of fluorescent antibody. The first of these, infectious canine hepatitis, is a disease of dogs and foxes, first described under this name by Rubarth in 1947. The typical inclusion bodies of this disease had been encountered in two dogs and described

by Cowdry and Scott (1930), forming one of the bases of their original description of the Type A intranuclear inclusion. In the livers of experimentally infected dogs, various stages in the development of this inclusion body were encountered in the hepatic cells The first appearance of antigenic material was as a bright ring congruent with the nuclear membrane. Thereafter a few small antigenic particles appeared inside the nucleus, increased in number and finally filled a large portion of the central part of the nucleus During this development the nuclear membrane continued to contain viral antigen (Coffin, Coons and Cabasso, 1953)

A careful study of the development of the herpes simplex inclusion in a pure line strain of cancer cells, derived from a human epidermoid carcinoma of the larynx, has been carried out by Lebrun (unpublished) She followed the changes in antigenic material in tissue culture with time, comparing the results with those obtained by staining with Giemsa and with haematoxylin and eosin. The findings were consistent with those of Crouse, Coriell, Blank and Scott (1950) and of Scott, Burgoon, Coriell and Blank (1953) As noted above the first detectable antigen made its appearance as a small fluorescent sphere inside the nucleus This was followed by gradual accumulation of antigenic material in strands and spheres within the nucleus, until finally antigenic material filled the whole nucleus At this stage, the nucleus was evidently filled with antigenic material, but the typical Type A inclusion had not yet made its appearance. Later, antigenic material began to appear in the cytoplasm and gradually disappeared from the nucleus until in the end-stages the cytoplasm was brightly [illegible]

coverslips stained with haematoxylin and eosin.

The third viral inclusion which has been investigated by means of fluorescent antibody is that produced by canine distemper. In recent investigations carried out in ferrets and

dogs by Liu and Coffin (unpublished) a close correlation has been found between the appearance of objects containing antigen and the staining of eosinophilic inclusions by Seller's method. When smears from the bladders of infected dogs are stained by a method commonly used in veterinary pathology, the S-3 stain of Shorr (Page and Green, 1942), the red objects visible in the cytoplasm are usually not antigenic, although barely visible objects stained bluish by this stain contain antigen. There is evidence, therefore, in the case of canine distemper that the inclusion bodies contain antigen, and that infection by this virus also produces changes in the cell which are tinctorially demonstrable but which do not contain antigen.

Lebrun's study of herpes simplex inclusions indicates, as suggested by Scott *et al.* (1953) on the basis of the Feulgen reaction, that the classical inclusion body is a "morphological residuum" containing little or no virus. There are indications in the case of canine infectious hepatitis and in the case of canine distemper that morphological residue may also be present after the viral infection in the cell has run its course. The answer to the old question of whether inclusion bodies represent aggregations of virus can evidently be given for these cases. During the early development of an inclusion, viral antigen and therefore presumably active virus is present. When the infection has run its course, inclusion bodies sometimes represent what might be called cytological scars and no longer contain virus particles.

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DISCUSSION

Coons: In the case of the ferret, where there is the nuclear staining, it doesn't seem to be virus multiplication, but the diffusion of a small particle from the cytoplasm into the nucleus In the case of the chick

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Bang: I didn't, of course, mean to infer that the data on vaccinia, shall we say, or on the beautiful intranuclear inclusions were subject to this thought; my question more concerned the presence of material early in infection.

Coons: In the very early tests, in the case of herpes it looks pretty clear, but in the case of influenza we would not want to say that it is [illegible]

eable, as it seems to be; in the first place there are electron photomicro- [illegible]

wash out on nuclear isolation.

[illegible]

Williams I was wondering if some of the narrow band-pass filters might allow you to clean up the background considerably by passing [illegible]

or sooner or later than extracts become infective?

Coons We cannot see the antigen in a single virus particle

Bawden You haven't assayed by the two methods?

Coons No, we haven't

an aliquot of the conjugate with this pellet, he removed the nuclear staining of the heterologous serum and most of its cross-reaction as well If he stained an FM1 infected membrane with FM1 conjugate, the

specifically in these cells.

Bang: Have you had a chance to study any of the recently adapted strains of influenza that produce filaments?

Coons: No, I don't think so The only strains that Liu has studied are PR8, FM1, and a strain that Dr. Finland isolated from a House Officer, in 1942 I think it was, which was a local strain, simply called Farrington, closely related to PR8.

Bang: None of the FM1, for instance, was studied in the chick embryo?

Coons: It was all studied in the ferret.

Bang: In tissue culture preparations, of course, the cell itself is flattened so that a thin section through the nucleus which is studied in electron microscope frequently shows very deep indentations in the nucleus which contain projections of the cytoplasm. I wondered whether in some of your nuclear staining you are not in reality dealing

process.

The second thing it might be is movement as the section thaws The sections are cut frozen, put on a cold slide, and then thawed, and of course the body water of the mouse, or whatever animal it is, moves

THE USE OF RADIOACTIVE INFLUENZA VIRUS TO DETERMINE THE FATE OF THE INFECTING PARTICLE ON ENTRY INTO THE HOST CELL

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By cultivation in eggs in which radioactive sodium phosphate has been introduced into the allantoic fluid, the influenza virus can be labelled with radiophosphorus (Graham and McLelland, 1949; Hoyle, Jolles and Mitchell, 1954; Liu, Blank, Spizizen and Henle, 1954), and by cultivation in eggs containing radioactive methionine it can be labelled with radiosulphur. The degree of labelling which can be attained is limited by the toxicity of radiophosphorus and radiosulphur to the fertile egg. The egg will tolerate 1 millicurie of radioactive sodium phosphate and about 250 microcurie of radioactive methionine. With these doses labelled virus preparations can be made in which the Geiger count per minute per ml is of the same order as the haemagglutinin titre. The virus is freed from contamination with non-viral ^{32}P or ^{35}S by two cycles of adsorption-elution from red cells, the cells carrying the adsorbed virus being thoroughly washed at each cycle before elution

Radioactive virus prepared in this way has been used to determine the fate of the infecting particle on entry into the host cell. The D.S.P. strain of virus A was used Radioactive virus was introduced as a primary inoculum into the allantoic sac of 12-day fertile eggs, or into de-embryonated eggs, and after 1½ hr. incubation to allow entry of virus into the cells, the chorioallantoic membranes were collected. By making various types of extract an attempt has been made to determine the chemical state, particle size, and location of radioactive material in the cells. In order to attain the maximum possible

Bawden: How long after you inoculate tissues with a large dose of virus can you still identify any of it?

Coons: In the case of influenza, within three hours.

Stoker. If you compare it with a sensitive complement fixation test can you detect antigen sooner?

Coons: You can detect a very small absolute amount of antigen, provided it is concentrated. If you have one infected cell you can find it, which you could not do with any other method. But if the concentration is not high, you cannot see it even if there is a lot of stuff there.

Spooner: How does the sensitivity of the antiglobulin method compare with the earlier one?

Coons: I cannot give you a quantitive answer, but it is considerably

so far shown any discrepancies as regards what is known about interference between viruses? And is she planning to use this technique to

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By cultivation in eggs in which radioactive sodium phos-hate has been introduced into the allantoic fluid, the in-uenza virus can be labelled with radiophosphorus (Graham nd McLelland, 1949, Hoyle, Jolles and Mitchell, 1954; Liu, lank, Spizizen and Henle, 1954), and by cultivation in eggs ontaining radioactive methionine it can be labelled with adiosulphur. The degree of labelling which can be attained s limited by the toxicity of radiophosphorus and radio-ulphur to the fertile egg. The egg will tolerate 1 millicurie of adioactive sodium phosphate and about 250 microcurie of adioactive methionine. With these doses labelled virus preparations can be made in which the Geiger count per minute per ml. is of the same order as the haemagglutinin titre. The virus is freed from contamination with non-viral ^{32}P or ^{35}S by two cycles of adsorption-elution from red cells, the cells carrying the adsorbed virus being thoroughly washed at each cycle before elution

Radioactive virus prepared in this way has been used to determine the fate of the infecting particle on entry into the host cell. The D S.P. strain of virus A was used. Radioactive virus was introduced as a primary inoculum into the allantoic sac of 12-day fertile eggs, or into de-embryonated eggs, and after 1½ hr. incubation to allow entry of virus into the cells, the chorioallantoic membranes were collected. By making various types of extract an attempt has been made to determine the chemical state, particle size, and location of radioactive material in the cells. In order to attain the maximum possible

uptake of radioactive material it was necessary to use very large inocula, and a dose of 1,000 haemagglutinin units per egg has been used. With this dose about 40 per cent of the inoculated virus is taken up by the cells.

Properties of radioactive virus

When virus is labelled with ^{35}S the whole of the radioactivity of the final purified virus preparations is carried by the virus particles. It can be completely removed by adsorption with red cells, by centrifugation for 1 hour at 26,000 *g*, or by precipitation with protein precipitants such as 5 per cent trichloracetic acid or 50 per cent saturated ammonium sulphate. With ^{32}P about 95 per cent of the radioactivity of the final preparation is carried by the virus, the remaining 5 per cent being contaminating radioactive phosphate. This is due to the fact that radioactive sodium phosphate enters rapidly into the red cells used in the purification cycles and is more slowly released, so that some inorganic phosphate is carried over to appear in the final product.

Purified influenza virus contains about 60 per cent protein, 35 per cent lipid, 3·5 per cent carbohydrate, and 0·74 per cent ribonucleic acid. Radiophosphorus is incorporated in the lipid and the nucleic acid in proportions which vary according to the technique used in making the labelled virus (Hoyle and Frisch-Niggemeyer, 1955). With different preparations the lipid ^{32}P ranges from 20–40 per cent and the nucleic acid from 60–80 per cent of the total. When purified influenza virus preparations are shaken with ether the virus disintegrates with the release of separate haemagglutinating and complement fixing "soluble antigen" particles. The whole of the nucleic acid ^{32}P of the virus appears in the soluble antigen fraction. With radiosulphur the whole of the virus protein is labelled and both haemagglutinin and soluble antigen fractions contain ^{35}S.

Technique of Geiger counting

With radiophosphorus the most satisfactory assessment of the radioactivity was obtained by counting 1 ml. fluid samples

which were placed in shallow metal dishes beneath a Geiger counter of the end window type. In effect, fluid samples were counted at infinite thickness. With fluids of different density a correction may be necessary for self-absorption in the sample, but with most of the fluids used this correction was so small as to be negligible

Liquid counting is unsatisfactory with radiosulphur as, owing to the low energy of β particles emitted from ^{35}S, self-absorption in the fluid is very great. Drying the sample produces a 36-fold increase in the count Dry counting however introduces several technical difficulties. Preparations must not have an acid reaction or ^{35}S may be lost as hydrogen sulphide during drying Irregular drying is also a problem. It was found best to dry 0 5 ml samples in metal dishes which had been polished with metal polish to give a non-wettable surface to prevent spreading of the fluid. With dry counting the corrections for self-absorption in different types of sample were large. These corrections were determined by measuring the count given by known amounts of radioactive methionine included in the various types of fluid before drying

Experiments with virus labelled with radiophosphorus

Fertile eggs were inoculated with 1,000 haemagglutinin units of D.S.P virus labelled with ^{32}P. After 1½ hr. incubation the chorioallantoic membranes were collected. It was found that 40 per cent of the inoculated virus was taken up by the cells. The membranes were frozen and thawed three times and a saline extract made using 1 ml. of saline per membrane. In different experiments from 20–40 per cent of the total ^{32}P of the infected membranes was recovered in the extract. This ^{32}P was not present as unaltered virus since the infectivity of the extracts was very low and it contained no demonstrable haemagglutinin. Most of the ^{32}P present in the extract was not precipitated by protein precipitants, and 80 per cent of it remained in the supernatant after centrifugation for 4 hours at 108,000 g. The chemical state of the ^{32}P in the extract was

determined in the following way. A sample was dried and extracted with ethanol-ether to determine the phospholipid ^{32}P. A sample was precipitated by half-saturation with ammonium sulphate, the precipitated ^{32}P representing nucleoprotein + lipoprotein. A further sample was precipitated with 2 volumes of ethanol, the precipitate representing nucleoprotein + nucleic acid, while the supernatant activity was taken to be due to phosphorus compound of low molecular weight + lipid. The average result of four such experiments indicated the following distribution of ^{32}P in the saline membrane extract.

Lipid	2 %
Nucleoprotein	12 %
Free nucleic acid	29 %
Low molecular weight phosphorus	57 %

It is clear from these results that on entry into the host cell the virus particle becomes disintegrated. Detailed results of the work with radiophosphorus have recently been published (Hoyle and Frisch-Niggemeyer, 1955).

Fate of the virus phospholipid

About 30 per cent of the ^{32}P of labelled virus is present as ethanol-soluble phospholipid. The content of phospholipid ^{32}P of 1½ hr. infected membranes is much less than 30 per cent. A saline extract of the membranes contains only 2 per cent lipid ^{32}P, and when the residual membranes were dried and extracted with ethanol their content of phospholipid ^{32}P was only 10–20 per cent. From 60–80 per cent of the original virus lipid ^{32}P was no longer recoverable as such from 1½ hr. infected membranes. Disintegration of the virus lipid would be expected to result in the appearance of ^{32}P in the form of phosphorus compounds of low molecular weight, and it seems probable that the low molecular weight ^{32}P found in saline extracts of 1½ hr. infected membranes is derived from the virus lipid.

Fate of the virus nucleoprotein

Part of the ^{32}P present in 1½ hr. membrane extracts appears to be present as free nucleic acid, suggesting that on entry into the cell the virus nucleoprotein is disrupted. However, the major part of the virus nucleoprotein ^{32}P does not appear in saline membrane extracts but remains in the residual membranes and must therefore be associated with some insoluble cell constituent. It seems probable that this constituent is the cell nucleus, since a large amount of ^{32}P can be recovered from the residual membranes by extraction with molar sodium chloride solution in which the cell nuclear material is soluble. The ^{32}P present in such molar sodium chloride extracts is partly precipitated along with the deoxyribonucleoprotein on dilution with water and is partly present as free nucleic acid.

The overall recovery of ^{32}P from 1½ hr infected membranes is illustrated by the following experiment. The membranes were frozen and thawed three times and then three successive extracts were made with physiological saline, followed by four successive extracts with molar sodium chloride. Three extracts were then made with ethanol and the final residue dissolved in normal sodium hydroxide. Geiger counts gave the following result

1st physiological saline extract	106	118 c.p.m.
2nd physiological saline extract	12	
3rd physiological saline extract	0	
1st molar NaCl extract	69	138.5 c.p.m.
2nd molar NaCl extract	48	
3rd molar NaCl extract	21.5	
4th molar NaCl extract	0	
1st ethanol extract	13	75 c.p.m.
2nd ethanol extract	62	
3rd ethanol extract	0	
Residue in N-NaOH		213 c.p.m.

Of the total membrane ^{32}P, 22 per cent was extracted by physiological saline, in the form of phosphorus compounds of

low molecular weight, nucleic acid, nucleoprotein and a trace of lipid. 25 per cent was extracted by molar sodium chloride, this material consisting of nucleoprotein and nucleic acid. Ethanol extracted 14 per cent as lipid. The residue after all the extractions still contained 39 per cent of the ^{32}P in unknown state.

Experiments with virus labelled with ^{35}S

When de-embryonated eggs were inoculated with virus labelled with ^{35}S and saline extracts made from the chorio-allantoic membranes, after 1½ hr. it was found that some 25 per cent of the total membrane ^{35}S was recovered in the extract. This material was not intact virus since it did not agglutinate red cells and its infectivity was only 1 per cent of that which could have been expected from its content of ^{35}S. About 65 per cent of the ^{35}S in the extract was not precipitable

presence indicates hydrolysis of virus protein in the cell. Some of this low molecular weight ^{35}S diffuses out of the cells during incubation of the infected eggs and appears in the roller fluid. The remaining 35 per cent of the ^{35}S in the membrane extract was present as protein of large particle size since it could be sedimented in 1 hr. at 26,000 *g*.

When 1½ hr. membranes were serially extracted with physiological saline the ^{35}S recovered in successive extracts fell rapidly in the early extracts to a low level, which was then maintained over a long series of extracts. When physiological saline was replaced by molar sodium chloride there was a slight increase in the recovery of ^{35}S but again the amount recovered remained constant over a long series of extracts. The ^{35}S in the early saline extracts consisted of amino acid and large particle size protein. In the later extracts all the ^{35}S recovered appeared as high molecular weight protein. Even after 12 successive extracts the residual membranes still

contained some ^{35}S. A typical experiment is given below. After 3 saline extracts the membrane residues were divided and with one part the extractions were continued with saline and with the other molar sodium chloride was used.

1st physiological saline extract	66 c.p m.		
2nd physiological saline extract	17 4 c p m.		
3rd physiological saline extract	8 8 c p m.		
4th physiological saline extract	6·4	4th extract molar NaCl	10·1
5th physiological saline extract	8·0	5th extract molar NaCl	5 6
6th physiological saline extract	3 6	6th extract molar NaCl	15·7
7th physiological saline extract	2 6	7th extract molar NaCl	6 2
8th physiological saline extract	1 8	8th extract molar NaCl	4 8
9th physiological saline extract	3 4	9th extract molar NaCl	7 3
10th physiological saline extract	2·2	10th extract molar NaCl	0 5
11th physiological saline extract	3 0	11th extract molar NaCl	5·3
12th physiological saline extract	2·0	12th extract molar NaCl	1·7
Residue	71	Residue	21

The results suggest that, on entry into the cell, part of the virus protein is hydrolysed to amino acid but the major part becomes associated with some insoluble cell component from which it can be slowly extracted by physiological saline and more readily by molar sodium chloride. There is no evidence of any specific association of ^{35}S with the cell nucleus such as was found with ^{32}P The slowly extractable protein was not infective virus since, although the extracts were infective, the infectivity was only 1/50th of that which might have been expected from the ^{35}S content.

Discussion

The experiments described here show that on entry into the cell influenza virus particles are disrupted. The virus phospholipid is attacked, with the appearance of water-soluble phosphorus compounds of low molecular weight. Disruption of the virus nucleoprotein results in the appearance of free nucleic acid and amino acid in cell extracts. With both ^{32}P-labelled and ^{35}S-labelled virus the major part of the

labelling is found in association with insoluble cell constituents but whereas the ^{35}S-labelled virus protein appears to be linked to a cytoplasmic constituent, the ^{32}P-labelled nucleic acid is largely found in association with the cell nucleus.

The interpretation of the results is rendered difficult by the fact that virus disintegration products may be immediately used for synthesis, and it is possible, for example, that the amount of free labelled amino acid present in cell extracts does not accurately reflect the amount of protein hydrolysis which has occurred. Similarly, the finding that with ^{32}P-labelled virus the cell nuclear DNAP is labelled may mean either that virus RNA has linked to the nuclear DNAP, or that small molecular weight ^{32}P derived by disintegration of the virus lipid has been used for synthesis of DNA.

Studies of the structure of influenza virus particles have indicated that they probably consist of a packet of soluble antigen and haemagglutinin particles enclosed in an envelope of lipoprotein and mucoprotein derived from the wall of the host cell. It may be that on entry into the cell the virus envelope and the haemagglutinin remain on the cell membrane while the nucleoprotein soluble antigen particles enter the cell and are disrupted, with the appearance of amino acid and of free nucleic acid which enters the cell nucleus.

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DISCUSSION

Dulbecco: You have not discussed the possibility of the incorporation of the small molecular phosphorus obtained from degradation of the phospholipids into the RNA by chemical methods; this would be an artifact.

Crick: Could you not take the small material and add it to a quite different cell and see if it is then incorporated?

Hoyle: I rather think it would not go in at all. You have got to have a receptor.

Watson: Dr. Hoyle, what percentage of the particles which you had in your preparation did you believe to be infective?

Hoyle: It was a standard virus, so that we would say of the order of

there is some haemagglutinin. You have got to have the intact virus particles to demonstrate it, and all the evidence that I know of suggests that it is a sort of receptor substance on the surface, and how haemag-

Hoyle: I am not suggesting that the haemagglutinin is right in the middle. We think the soluble antigen is in the middle, and the haemagglutinin is in a layer outside that. But outside the whole of it, we feel that there must be some sort of membrane. And, in fact, the electron microscope pictures do rather suggest the existence of such a membrane round the particle

Smith: Yes, but the membrane may be formed of what we term the agglutinin receptors. Is there any evidence whatsoever for the existence of a membrane as a separate structure, apart from the reactive

discrepancies

labelling is nothing like high enough. To give you an idea of how low it is, we calculate that there is one atom of radiophosphorus in fifty virus particles

Isaacs: Dr. Dulbecco, have you done any exclusion experiments with influenza?

Dulbecco: No.

of course, they will not effect the result, because we are dealing with the cells and not with the allantoic fluid

Dulbecco: We don't know what happens at the moment of exclusion; maybe they are broken down. But we don't know for certain

Gard In the elution or extraction experiments, the sulphur distri-

remained more or less intact on the cell surface

your material wet and just use alcohol.

Pirie It goes better still if you take it to pH 1 and then extract

Ada Dr Hoyle, after you extract your wet virus with alcohol-ether how much phosphorus is left in the virus particles? How much nucleic acid does it correspond to?

Hoyle You mean a chemical estimation?

Ada Yes.

Hoyle I don't know.

Ada Because in our experiments, although admittedly we do use dry virus, we found that chloroform-methanol would extract far more phospholipid than alcohol-ether

Knight. Dr. Hoyle, in your fractionation do you find that the envelope and the haemagglutinin both contain mucoprotein?

Hoyle: Yes.

Knight· From which I assume that you find the polysaccharide component in each of these constituents?

Hoyle Yes.

Knight: Do you know what proportion of polysaccharide is in these two?

Hoyle· According to our measurements, the intact virus contains 3·5 per cent carbohydrate, which is rather lower than the figure which you got originally The haemagglutinin contains 4 per cent, but as far as we can estimate the haemagglutinin only makes up about 15 per cent of the whole particle, so that the major part of the carbohydrate is not in the haemagglutinin at all We have done carbazole curves, both on the intact virus and on the haemagglutinin With the intact virus you get a very irregular curve which cannot be matched by any single mixture of sugars But with the haemagglutinin the carbazole curve is much simpler and matched well to an equimolar mixture of galactose and mannose. It was rather like one of the preparations that you got in your original work.

Knight A much simpler method of sugar analysis, which we are now using, is simply to hydrolyse with N-HCl and then use ion exchange to get rid of a lot of things, and then run your sugars using paper chromatography. In this way we have found, and Mr Ada has also, that the influenza polysaccharide contains fucose as well as the components which were originally reported Also the amino sugars, glucosamine and galactosamine both seem to be present, whereas only glucosamine was reported originally. In many respects, the polysaccharide seems to be very similar to the blood group substances.

Pirie Isn't there any sulphate ester?

Knight· We haven't looked for that.

Lwoff. Dr. Hoyle suggested that the genetic material of the virus penetrates into the nucleus and is then bound to the nucleic acid of the cell. Is it known whether it is bound to the DNA or to the RNA of the cell?

Hoyle· I only suggested that possibly it could happen. The only thing I would say is that I do think there is some evidence that the nucleic acid goes into the nucleus

You have to remember the very small amounts of material that we are handling, and there is a limit to what you can practically do. The chemical analysis is not good enough. If we could only label more heavily it would be very much easier, but you may not perhaps realize that with some of our samples we are counting already for half an hour, which is a long period of time to count in a Geiger counter. Now, if you have got 10 or 20 samples, and each one of them has to be counted for half an hour, you have got a pretty full day's work. If you leave them

[illegible]

[illegible]: Our more highly labeled virus was produced in tissue culture.

Ada How many times the background is your count?

Hoyle: [illegible]

[illegible] about [illegible]0 per cent

Watson Not if the drying is uniform so that self adsorption can be taken into consideration

Hoyle Yes, but not in any convenient time.

THE SIZE DISTRIBUTION OF SPECIFIC ANTIGENS IN VIRUS-INFECTED TISSUES AND THEIR SIGNIFICANCE

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C S I.R and U.C.T. Virus Research Unit, Department of Pathology, University of Cape Town

WITH few exceptions animal viruses can be detected in emulsions of virus-infected tissues only by titration of infectivity, by serological methods such as complement fixation, and by electron microscopy. In exceptional cases, of which influenza is the classical example, *in vitro* methods such as haemagglutination are available.

In most cases we know relatively little about the occurrence of virus particles not detectable by the usual tests for infectivity, and we cannot therefore study them effectively This paper presents some further evidence that fully infective virus particles can vary in readily detectable properties. The one we have studied is that of size. It is the purpose of this paper to draw attention to the possible significance of the size variation and through discussion to seek guidance for further work.

Antigens, other than those in the virus particles themselves, are demonstrable by complement fixation in many virus infected tissue suspensions. These so-called soluble antigens have for example been obtained from tissues infected with vaccinia virus (Craigie, 1932; Craigie and Wishart, 1936). These authors conclude that soluble antigen is a surface constituent of the vaccinia virus. The vaccinia soluble antigen is capable of stimulating formation of antibodies which not only precipitate soluble antigen but will agglutinate elementary body suspensions. Extensive studies made with influenza viruses have shown that soluble antigen can be detected by complement fixation in chorioallantoic membranes from

influenza-infected eggs before the appearance of haemagglutinin or fully infective virus. Hoyle's findings (1950, 1952) and the results of electron photomicrographic studies of soluble antigen from virus treated with ether (Hoyle, Reed and Astbury, 1953) have led to the conclusion that soluble antigen of influenza virus is the fundamental multiplying unit during intracellular synthesis and that it is an essential constituent of the virus particle. Ada and co-workers (1953, 1954) have undertaken serological and chemical investigation on soluble antigen from lungs of influenza-infected chick embryos. More recently Wildy and Holden (1954) have demonstrated the occurrence of soluble antigen in association with herpes virus, and Randrup (1954) has studied complement fixing antigens of small particle size in association with foot and mouth disease virus.

In this laboratory attention has been paid particularly to the soluble antigens encountered in suspensions of brains from suckling mice infected with poliomyelitis, blue tongue, rabies, Rift Valley fever and horse sickness viruses. Although some attempts have been made to elucidate the nature of the soluble antigens, most attention has been devoted to investigations of their serological specificity and to measurements of their particle sizes in comparison with those of the infective virus particles.

Material and Methods

Particle size determinations were made by a centrifugation method using a Spinco preparative centrifuge (Polson and Linder, 1953), by a method based on diffusion measurements (Polson, 1948), and in some cases particles of different sizes were separated by differential migration into agar gels of appropriate concentrations (Polson, 1956)

Most of the virus strains used were adapted in this laboratory to the brains of baby mice. Mice varying in age from 2–4 days (for blue tongue and rabies viruses) to 4 or 5 days (for MEF_1 strain of poliomyelitis) were used

Soluble antigens were separated from virus antigens by

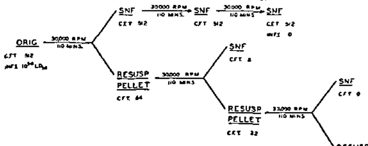
simple centrifugation of whole brain suspensions in phosphat buffer or brains previously extracted in acetone and ethe according to Casals' (1949) method.

Complement fixation tests were carried out according to th *method of Casals and Olitsky (1950)* or *that of Fulton an* Dumbell (1949).

Results

Relative amounts of soluble antigen and virus detectabl in brain suspensions

The most clear-cut results have been obtained with MEF poliomyelitis. Crude brain suspensions with complement fixing and infective titres of 1/512 and $10^{5\cdot3}$ respectively ca be separated by centrifugation into two fractions—the firs containing predominantly soluble antigen and the other pre *dominantly virus The soluble antigen in a typical experimen* fixed complement to a dilution of 1/512 but contained no viru detectable in tissue culture or by intracerebral inoculation o mice. The virus fraction, on the other hand, had a complemen fixing titre of only 1/32 and an infective titre of $10^{4\cdot8}$ (Table I)







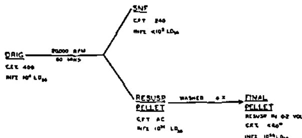
Although the methods employed cannot be regarded as quantitative, the evidence is sufficient to suggest that the complement fixation is predominantly a function of the soluble antigen. These results were substantiated also in the case of rabies and blue tongue (Table II)

TABLE II. The separation of rabies soluble antigen from infective virus in suspensions of suckling mouse brains in M/15 phosphate buffer.

C.F.T. = Complement fixing titre expressed as the reciprocal of the highest dilution giving 50 per cent fixation in the presence of 1/20 mouse immune serum.

Infy = Virus titre expressed as log LD_{50} per ml for 3–4 weeks old mice

Washing consisted of resuspension of deposit in original volume of buffer and recentrifugation at 20,000 r p m for 1 hour



* Crude resuspended deposit markedly anticomplementary The titre of 60 was obtained with material centrifuged at 10,000 r p m for 10 minutes.

Purified polio virus is readily inactivated during slow drying. Such virus which has been subjected to prolonged dialysis followed by drying has been shown by electron photomicrography to break up into small spherical particles with an average diameter of 10–12 mμ. (Hampton, 1955) (Fig. 1) and which appear to be arranged in a regular almost crystalline form. Such dissociated virus is however inactive as antigen in *in vitro* tests. This suggests, though it does not prove, that the soluble antigen responsible for the specific complement

those which we have studied, this virus therefore offers the best opportunity of determining the relative rates of production of soluble antigen and infective virus.

In a typical experiment, the first new virus appeared 14–24 hours after its intracerebral inoculation into infant mice. Soluble antigen, on the other hand, was first detected after 24–39 hours. The rate of increase of soluble antigen thereafter appeared to parallel the multiplication of virus. Antigen detectable by complement fixation certainly did not precede the formation of virus under the conditions of our experiment.

Particle size distribution of infective virus

In the case of several smaller viruses that we have investigated, it has been possible to show by centrifugation methods that infective particles occur in definite size groups. Thus in the case of suckling mouse adapted MEF_1 poliomyelitis infective particles, with average diameters of 24 and 30 mμ respectively, have been identified. The former comprises only 1 per cent or less of the total virus population. It has been possible to identify virus particles of both dimensions in approximately the correct proportions in electron photomicrographs, and the 24 mμ. particles can be separated from the 30 mμ. particles by an agar gel migration method. The main value of the agar diffusion experiments is to demonstrate that the difference detected by centrifugation is dependent on actual size and not on differences in specific gravity resulting from adherence of lipoids to the apparently smaller particles. Suckling mouse adapted MEF_1 virus when inoculated into adult mice has in two experiments, so far performed, stimulated the formation only of 24 mμ. particles, which is not the normal size of the MEF_1 virus when it has been adapted to adult mice only (Fig. 2).

It is of particular interest that the infective titre of the suspensions of the brains of adult mice, infected with MEF_1 virus which has never been passaged in sucklings, is lower than that of suckling mouse brain suspensions. Centrifugation has

revealed the presence of infective particles which are entirely or predominantly 30 mμ. in diameter. There may be a small proportion of smaller infective particles not detectable by the centrifugation method in material of such low titre.

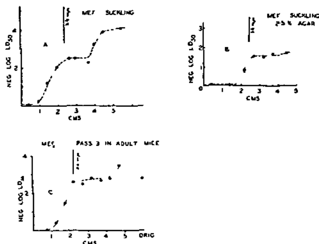


FIG 2 Sedimentation of MEF_1 virus in emulsion of suckling mouse brain by centrifugation at 20,000 r p m. for 100 minutes in the presence of C. sincta haemocyanin which has an average particle diameter of 24 mμ

(A) Crude suspension in M/15 phosphate buffer
(B) Fraction separated by migration into 2 5 per cent agar
(C) Suspension of adult mouse brains of the third passage after IC inoculation of "suckling" virus.

Studies with other viruses carried out in this laboratory have confirmed the occurrence of particles of different dimensions in suspensions of tissues infected with single viruses. The finding of different sedimentation constants by Melnick *et al.* (1951) in their experiments on Coxsackie, Lansing and Theiler's viruses subjected to different periods of ultracentri-

those which we have studied, this virus therefore offers the best opportunity of determining the relative rates of production of soluble antigen and infective virus.

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that they do remain in the supernatant fluid. They are in fact particulate, occurring in sizes of approximately 8–12 mμ or larger. Our experiments so far have revealed that in the case of poliomyelitis, rabies and yellow fever, neurotropic Rift Valley fever and horse sickness viruses, the soluble antigens have an approximately uniform particle size

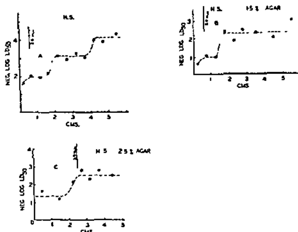


FIG 4 Sedimentation of horse sickness virus by centrifugation at 11,000 or 20 000 r p m. for 100 minutes C sincta haemocyanin boundary (24 mμ) is shown

(A) Crude suspension in M/15 phosphate buffer. 11,000 r p m. for 100 minutes.

(B) Fraction separated by migration into 1 5 per cent agar 11,000 r p m. for 100 minutes

(C) Fraction separated by migration into 2 5 per cent agar 20,000 r p m for 100 minutes

In the case of the blue tongue viruses, however, complement fixing particles intermediate in size between 8 mμ. and that of fully infective virus have been found. The results especially with blue tongue suggest that the soluble antigen is either polydisperse, being made up of particles which may be aggregates, or associated with a variety of tissue components including lipids.

particles in their experiments, their results can be interpreted as showing the existence of 24 and 30 mμ. particles (see Selzer and Polson, 1954).

Further evidence that the size distribution of virus particles may depend on adaptation, as in the case of MEF_1 virus in infant mouse brains, was obtained with Rift Valley fever virus. The neurotropic variant after repeated passages in mice and eggs occurs in virus-infected mouse brains in 30 mμ. and 50 mμ. particles, but virus adapted to mice only has revealed the presence of 50 mμ. particles alone (Fig. 3). The pantropic strain in virus-infected mouse blood also occurs in the large form only (Naudé, Madsen and Polson, 1954).

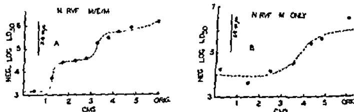


FIG. 3. Sedimentation of neurotropic Rift Valley fever virus by centrifugation at 11,000 r p m for 100 minutes C. sincta haemocyanin boundary (24 mμ) is shown.

(A) Virus adapted by repeated passages in mice and eggs.
(B) Virus adapted by passage in mice only

In the case of horse sickness virus infective particles with diameters of 24, 30 and 50 mμ. have been found. With this virus it has been possible to separate the two smaller components by migration into 1·5 per cent agar, and the 24 mμ. particle alone by migration into 2·5 per cent agar (Fig. 4).

Particle size determination of the larger viruses (rabies and blue tongue) by the methods employed in this laboratory have been unsatisfactory.

Particle size of soluble antigens

The antigens detectable by complement fixation and which remain in the supernatant after infective virus particles have been removed by centrifugation, are soluble only in the sense

small units occurs, whereas aggregates of various sizes are present in crude saline extracts.

Diffusion measurements of soluble antigens have confirmed the results of centrifugation experiments in that the majority

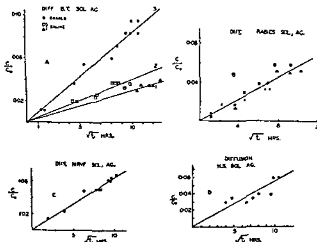
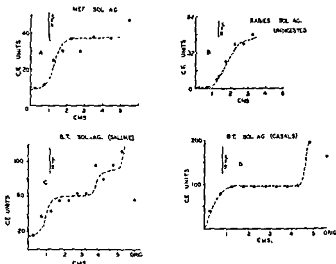


FIG. 6 Graphs showing diffusion rates of soluble antigens in suckling mouse brain suspensions.

of soluble antigens appear to occur as particles which approximate 8 to 12 mμ. in diameter (Fig 6)

In the case of the MEF_1 strain of polio virus adapted both to adult and suckling mice, it is of particular interest that soluble antigen recognizable by complement fixation is either absent in the adult or present in a different form to that in

In contrast, blue tongue soluble antigen from brains extracted with acetone and ether by the Casals method is of uniform particle size $\pm$ 8 mμ as determined by centrifugation



[illegible]

(A) Soluble antigen of MEF_1 poliomyelitis virus in suckling mouse brains.

[illegible]

(Fig. 5). Though the total yields of soluble antigen in suspensions prepared by saline extraction and acetone-ether extraction are similar, that in the latter type of suspension is uniformly of the smallest dimension. It may be that under appropriate conditions such as are attained by acetone-ether extraction, disaggregation of complex particles into basic

small units occurs, whereas aggregates of various sizes are present in crude saline extracts.

Diffusion measurements of soluble antigens have confirmed the results of centrifugation experiments in that the majority

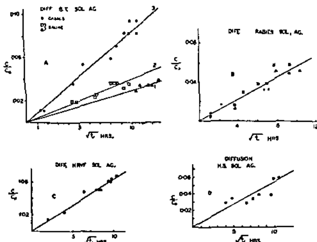


FIG 6 Graphs showing diffusion rates of soluble antigens in suckling mouse brain suspensions

of soluble antigens appear to occur as particles which approximate 8 to 12 mμ. in diameter (Fig. 6).

In the case of the MEF_1 strain of polio virus adapted both to adult and suckling mice, it is of particular interest that soluble antigen recognizable by complement fixation is either absent in the adult or present in a different form to that in

suckling mice. Thus adult mice immunized by repeated intraperitoneal injection of polio virus-infected suckling mouse brains produce antibodies that fix complement with antigens freed of virus (soluble antigen) or with virus itself, but mice immunized against "adult" mouse virus produce antibody which fix complement only with the virus fraction and not the

[illegible]

shown as reciprocals.

4 = complete fixation, 0 = no fixation.

ANTISERUM	ANTIGEN	ANTIGEN DILUTIONS								
		4	8	16	32	64	128	256	512	C
MOUSE SERUM VS. SUCKLING VIRUS	ORIGINAL EMULSION	4	4	4	4	4	4	3	[illegible]	0
MOUSE SERUM VS. ADULT VIRUS		4	4	4	4	0	0	0	0	0
MONKEY SERUM VS. LANSING VIRUS		4	4	4	4	0	0	0	0	0
NIL		0	0	0	0	0	0	0	0	
MOUSE SERUM VS. SUCKLING VIRUS	VIRUS FRACTION AFTER REPEATED CENTRIFUGATION	4	4	4	2	0	0	0	0	0
MOUSE SERUM VS. ADULT VIRUS		4	4	4	0	0	0	0	0	0
MONKEY SERUM VS. LANSING VIRUS		4	4	4	0	0	0	0	0	0
NIL		0	0	0	0	0	0	0	0	
MOUSE SERUM VS. SUCKLING VIRUS	SOLUBLE ANTIGEN	4	4	4	4	4	4	0	0	0
MOUSE SERUM VS. ADULT VIRUS		0	0	0	0	0	0	0	0	0
MONKEY SERUM VS. LANSING VIRUS		[illegible]	0	0	0	0	0	0	0	0
NIL		0	0	0	0	0	0	0	0	

soluble antigen. Antibody to soluble antigen has also not been [illegible] sera of monkeys immunized [illegible]

[illegible]

is a complete antigen, and whether it has any significance in immunization against virus infection have so far given equivocal results. Practical difficulties arise in the separation of

soluble from virus antigen Thus, in the case of rabies-infected newborn mouse brain suspensions, which because of their high content of soluble antigen were chosen for the work, it was found impossible to free the suspensions of virus without at the same time losing the bulk of soluble antigen.

Whilst realizing the limitation of experiments carried out with imperfectly separated "soluble" and virus antigen fractions, a study has been made of antisera prepared against fractions separated by centrifugation and in which residual virus was killed by heat or phenol

A suspension of rabies-infected newborn mouse brains with an infective titre of $10^{6.8}$ and which fixed complement to a dilution of 1/2560 was separated by differential centrifugation, and repeated washing of the virus-containing deposit into two fractions: (a) soluble antigen with infective and complement fixing titres of 10^1 and 1/1600 respectively, and

(b) a virus fraction in which the corresponding titres were 10^3 and less than 1/240 The preparation of this fraction took several days, and during this time control virus suspensions not subjected to centrifugation showed a hundred-fold drop in titre. Taking into account dead virus, the suspension must have contained virus equivalent to a titre of at least 10^5.

Groups of mice were immunized against each antigen using multiple doses of the antigens which after heating at 56° C for 30 minutes or treatment with 0.5 per cent phenol were incorporated in an adjuvant containing lanolin, paraffin and M. phlei.

The results recorded in Table IV show that the antigens stimulated the development of complement-fixing as well as neutralizing antibodies The amount of antibody elicited by the "soluble antigen" fraction is as much as that elicited by the virus fraction or a mixture in equal parts of the two. In the case of the heated antigens, however, the amount of neutralizing antibody produced is small.

From the results it cannot be concluded whether the soluble antigen is a complete antigen in itself, or a hapten which is an effective antigen only in the presence of the small amount of residual virus present. It appears significant that

heating affects the capacity of the antigen to stimulate the formation of neutralizing antibody, without significantly affecting its power to elicit the formation of antibody detectable by complement fixation.

The relative non-specificity of the soluble antigens is well exemplified by the results obtained with a variety of blue

Table IV. The results show the antibody elicited in adult mice by fractions of rabies-infected suckling mouse brains

Sol. Ag. — [illegible]

ANTIGEN USED FOR IMMUNIZ	CF TITRE	NEUTRALIZATION TITRE
SOL AG PHENOLYSED	160	>320
VIRUS ,,	320	320
SOL AG + VIRUS ,,	140	320
SOL AG HEATED	160	<32
VIRUS ,,	240	8
SOL AG + VIRUS ,,	240	15

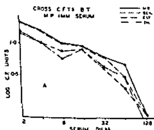
tongue viruses and with a group of horse sickness viruses. The results recorded in Table V show that striking differences between the blue tongue virus strains can be demonstrated by neutralization tests with mouse immune sera. The same sera were used in quantitative complement fixation tests (Fulton and Dumbell, 1949) using as antigens crude saline extracts of of virus-infected suckling mouse brains, or soluble antigens prepared from such brains extracted with acetone and ether. The results, of which typical examples are illustrated in Fig. 7,

TABLE V. Results of neutralization tests carried out with mouse immune sera against blue tongue virus strains adapted to baby mice The tests were carried out in 2–3 day old mice using the intracerebral route and 10 or 100 MLD of virus

+ to ++++ = increasing degree of neutralization,
0 = no neutralization

NEUTR TESTS B.T.

VIRUSES	SERA					
	CYP	TH	M P	EST	JAN	BEK
CYPRUS	++++	0	+	+++	+++	+++
THEILER	0	+++	0	0	0	0
MIMOSA PARK	+++	0	++	0	++++	++++
ESTANTIA	0	0	0	++++	0	0
JANSEN	++++	0	0	0	++++	+++
BEKKER	++++	0	0	+	+	+++



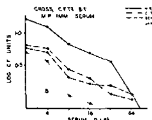


FIG. 7. Results of quantitative complement fixation tests with mouse

show that the soluble antigens of the different strains pre- [illegible]

extracts did suggest quantitative differences. Such crude antigens have in our experience, however, been difficult to standardize for use in quantitative tests. The differences are more likely to be dependent on unavoidable errors in standardization than on an increasing serological specificity of the particles intermediate in size between the smallest soluble antigens and the virus particles themselves.

Several of the soluble antigens which we have studied have been subjected to the action of certain physical and chemical agents, as well as a variety of enzymes in attempts to elucidate their nature. Thus the soluble antigen of rabies withstands a wide range of pH changes. At pH 4·3 the soluble antigen is completely precipitated from solution, but not all the antigen is recovered on resolution of the precipitate. The complement fixing power is unchanged by heating at 56°C for 30 minutes or by treatment with formaldehyde or phenol. Purified preparations of trypsin, RNase and DNase do not destroy complement fixing power of the soluble antigen completely but do reduce it to approximately 2/3 or 1/2 of the original.

Electrophoretic analyses have so far not shown significant differences between emulsions from rabies-infected suckling mouse brains and those from normal mice of the same age. Soluble antigen appears to be associated with the slowest moving components when electrophoresis is carried out in borate buffer at pH 8·6.

Discussion

Although absolute proof has not been obtained that the different sizes of infective virus particles as determined by centrifugation are not due to variable association with components from the tissue cells of the host, the experimental evidence does suggest that the differences are in the virus

particles themselves. Thus the dimensions as determined in repeated experiments are remarkably constant. In some cases, as with neurotropic Rift Valley fever virus, particles of uniform size may occur under certain conditions of adaptation, whilst under different conditions multiple sizes appear, even though tissue suspension used for the size determination by centrifugation is the same in both cases Furthermore, individual sizes can be separated by the use of agar gels of appropriate concentration. Electron photomicrography in the case of MEF_1 virus, which has been obtained in sufficiently pure form, has confirmed the differences shown by centrifugation.

The significance of the variations in size is at present unknown. That they may be related to process of adaptation is suggested by the results with Rift Valley fever virus Stanley has reported that different-sized particles in MEF_1 virus suspensions appear to differ in the time they take to produce paralysis in adult mice (Stanley, 1955, personal communication) It appears not unlikely that virus particles can exist in suspensions of infected tissues in various stages of "completeness", and that, in fact, non-infective forms equal in dimensions to or smaller than those recognizable by their infectivity may be present. It seems unlikely that the very constant virus particle size groups so far identified are entirely fortuitous. Thus amongst the animal pathogenic viruses the smallest infective particles are those of foot and mouth and lumpy skin disease virus (19 mμ.); poliomyelitis, Coxsackie and other viruses, including that recently isolated by Weiss *and referred to as Wesselsbron virus, occur as* 24 and 30 mμ. particles; horse sickness virus in dimensions of 24, 30 and 50 mμ. The relative volumes of the 19, 24, 30 and 50 mμ particles are approximately 1, 2, 4 and 16. It is not inconceivable that the larger MEF_1 particle contains the equivalent of 2 "minimal infective units" such as are present in the smaller variety, and that the largest horse sickness virus contains 4 or 8 smaller "minimal infective units". If this can be substantiated, it may be of great importance in relation

show that the soluble antigens of the different strains prepared by acetone ether extraction are indistinguishable, even [illegible]

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to virus structure and virus genetics. Unfortunately, size measurements by Polson's method of the larger viruses, such as that of influenza which have been extensively used in genetic studies, are unsatisfactory. However, it may soon be possible to study experimentally the significance of particle size in relation to genetic structure of the polio viruses and to degrees of their "completeness" or "incompleteness".

When trying to determine the nature and significance of soluble antigens in virus-infected tissues it is essential to keep in mind the results obtained with all those so far identified. But it is also important to realize that significant differences may exist between individual viruses, and even the same virus cultivated in different species. Information is still too scanty for definite conclusions, but certain hypotheses can be postulated. Their acceptance must await experimental proof.

In the case of influenza virus cultivated in eggs, one which has probably been studied in greater detail than others, the relative non-specificity of the soluble antigen and its early appearance during intracellular virus synthesis is well established. This non-specificity we have confirmed in the case of blue tongue and horse sickness soluble antigens. In rabies-infected mouse brains, though, we have not found that soluble [illegible] appears before the formation of infective virus. Soluble

antigen can be released from the surface of elementary bodies by appropriate treatment. In our experiments we have not succeeded in releasing soluble antigen from rabies virus which has been partially purified by ultracentrifugation or from MEF, polio virus purified by centrifugation and chloroform treatment. Such purified polio virus is still fully infective and is apparently homogeneous as determined by electron [illegible] microscopy; it can be dissociated by appropriate

allantois has been regarded as a virus "precursor", arguments have also been brought forward for regarding it as a "matrix substance" rather than an essential virus constituent. There is no conclusive proof that the vaccinia soluble antigen cannot be regarded as a "matrix" substance. Our own experience, and in particular the facts that MEF_1 soluble antigen is apparently present in suckling but not adult mouse brains and that disruption of purified MEF_1 virus does not release "soluble antigen", incline us to the view that the soluble antigen is not in all cases an essential virus component. The soluble antigens are nevertheless specific evidence of virus infection—even though their specificity is less marked than that of the elementary bodies themselves. The amounts in which they are present and their relative non-specificity in comparison with the virus particles with which they are associated, in themselves favour the hypothesis that they are characteristic by-products of intracellular virus synthesis rather than essential constitutents of the virus particles themselves.

Although the smallest particles associated with specific complement fixing activity are probably 8–12 mμ. in the case of the majority of viruses so far studied, results obtained in this laboratory with crude saline extracts of blue tongue-infected brain emulsions show that complement fixation is often associated also with larger particles intermediate between 8–12 mμ. and that of infective virus. The significance of this is doubtful. The disappearance of these intermediate particles in acetone and ether extracted emulsions suggests that they are due to mechanical association with tissue particles of various sizes and specific gravity or to the aggregation of varying number of the smallest particles The possibility cannot, however, be completely ruled out that they are stages of intermediate complexity in the synthesis of virus.

Whether the soluble antigen of influenza is antigenic has been placed in doubt by Henle (1953). On the other hand Ada and co-workers have shown that 'flu-infected chick embryo lung extracts freed of virus can stimulate antibody formation in rabbits. They have also made studies of the

RNA and DNA content of soluble antigen from 'flu-infected chick embryo lung extracts and conclude that DNA is probably not an essential constituent. The LS antigen of vaccinia is capable of stimulating the formation of precipitins and agglutinins in rabbits. In our experience the soluble antigen of rabies acts either as antigen, or specific hapten. It is of particular interest that phenol treatment preserves its ability to stimulate the formation both of neutralizing and complement fixing antibody. Heating on the other hand, whilst significantly interfering with the ability to evoke neutralizing antibody, leaves the power to stimulate formation of complement fixing antibody unaffected. Whether this difference between the effect of phenol and heat indicates the presence of two separate components or two functional groups of the same substance has not been determined. This evidence at first sight appears to be in conflict with the hypothesis that the soluble antigen is not an essential constituent of the virus particle. The relatively crude material used for immunization may, however, have contained antigens other than "soluble antigen" which were responsible for the production of neutralizing antibody and these may well be the heat-labile constituents present.

It is of interest to compare our findings with those obtained from the very much more comprehensive studies which have been made with bacterial viruses. In the case of phage-infected bacteria a variety of serologically specific components has been obtained from prematurely lysed cells. Thus the incomplete "doughnut" stage of phage can combine with complement fixing but not neutralizing antibody. An antigen of small particle size appears during the latter part of the latent period. It increases in amount prior to and in parallel with the increase in active phage. This antigen is probably responsible for the production of phage neutralizing antibody.

Although equally strong evidence for the occurrence of developmental stages of viruses in animal cells has not yet been obtained, there is evidence of the occurrence of a variety of antigens which differ in their reaction with complement

fixing and neutralizing antibodies in immune sera. The development of new techniques, such as the plaque counting method of Dulbecco (1952), will make it possible to apply to animal viruses quantitative procedures such as have been effectively used in the study of phages. It is hoped that this will assist in the elucidation of some of the problems which the work reported in this paper has revealed.

Acknowledgements

[illegible]

Funds.

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selves. It is not necessary to visualize the virus particles as being perfectly spherical.

Andrewes: What animal does that new virus that you mentioned infect?

v. d. Ende: Wesselbron is the name of the town where the virus was isolated. It is one that primarily infects sheep but it can also affect man I believe Smithburn has isolated a virus, which is probably identical, from mosquitoes. It has been isolated by Ondestpoort workers both

acid.

cannot easily give us, for example, how spherical these things are and whether they can be crystallized, as in the recent case of polio.

balance to obtain a dynamic equilibrium which gives you that sort of

on.*

* These remarks apply to objects with cubic symmetry The previous remarks refer to the *sub-units* of an object having cubic symmetry

DISCUSSION

Gard. Prof. van den Ende, have you considered the possibility that you have aggregates in your material? Did you try to break up aggregates mechanically or by other means?

[illegible]

from the CNS tissue of cotton rats In the stages of purification there did show up frequently a particle with a diameter of the order of 12 mμ, but which could be separated in a separation cell from the particle which turned out to have the infectivity—the nominal 30 mμ. particle that was found.

[illegible]

Andrewes: I am trying to place your work, Prof. van den Ende, in

[illegible]

expect to get such a regular series.

[illegible]

Williams: I realize that with respect to concentration gradient, it is the opposite way.

Crick: You haven't taken the same material and done the test at different dilutions? That would distinguish between whether it was two things or dynamic equilibrium

Watson: Have you tried the same experiment on plant viruses, say [illegible]

[illegible]

are 49 and 240. We could not decide what is the relationship between the two particles or whether both are infective. We could make crystalline preparations of the small particles and these were not very infective, but whether this means that only the big particles are infective or that, in getting them to the state of crystals, the small particles are inactivated is uncertain.

The more carefully that infections with plant viruses are studied, the more it becomes obvious that it is usual rather than exceptional for virus multiplication to lead to a variety of related products rather than to a single uniform product. In infections with some viruses, serologically related particles can be distinguished that differ in size, with others they differ in chemical constitution, and with still others they differ in both.

Crick: That was the material from which Mrs. Hodgkin obtained X-ray pictures?

Bawden. She got the X-ray pictures from crystals of the small particles.

Crick: And that definitely has the RNA in it, has it?

Bawden: Yes.

Crick: That does appear to be a sphere, although it has not been shown to have a cubic symmetry?

Bawden: Yes.

Pirie: The preparations we worked with at Rothamsted contained [illegible]

[illegible]

sedimentation velocity, which after all is the only thing measured, represents solely a difference of particle size? Would not this sole explanation be ruled out by the possibility that one particle contains nucleic acid, the other does not? Are not sedimentation velocities of the two components of turnip yellow mosaic virus comparably far apart?

Markham: Yes, 2 to 1.

v. d. Ende: It is, of course, important that in our experiments we are

Markham: In some of the early work at Duke University on the T-bacteriophages they showed two perfect boundaries under certain conditions of pH.

out on which these two were based. Is that an end-point titration?

v. d. Ende: The titrations were carried out on samples taken at half centimetre distances, dilutions were logarithmic and 6–10 mice used per dilution.

Dulbecco: Therefore the expected variability would be of the order of 0 3 or so logarithmically. I wondered, if we imagined the factor 0 3 of variability applied to the points in one of your slides, whether we would really be completely convinced that this is a step and not a continuous line.

v d. Ende: Yes, this has often been argued. An important fact is that these steps are so reproducible in repeated experiments. You can, of course, subject each titration to statistical analysis but you have also

er
o-

ve
ee
So far, we
n is that it
studied this

o apply, is Dr. Dulbecco's technique which will allow the offspring of single virus particles to be isolated and characterized

Watson: How do you rule out the possibility of convection when you are centrifuging?

v. d Ende: Dr Polson does the centrifugation in a haemocyanin gradient or in sucrose gradients. He has the added control in the haemocyanin giving a clear boundary.

Watson: But you would need very little convection to explain your results for you postulate *only one particle in a hundred of the smaller size*

concentration in the centrifuge cell It seems to me that

MORPHOLOGICAL ASPECTS OF VIRUS CELL RELATIONSHIPS IN INFLUENZA, MUMPS AND NEWCASTLE (*Myxovirus*)

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AND

A. ISAACS

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THIS paper will attempt to give morphological data concerning the infection of cells with several viruses of the *Myxovirus* group, i.e. the viruses of influenza, mumps and Newcastle disease (Andrewes, Bang and Burnet, 1955). There are three main stages at which infection may be studied, but only in the initial and final stages of infection of the cell is there any definite morphological evidence of virus activity.

Macrophages in tissue culture have long been known to engulf fluid droplets (Lewis, 1931) and this may be observed in thin sections in the electron microscope. Fig. 1 shows a section of a macrophage exposed to a high multiplicity of infection with N D V. Within the fluid droplets are masses of virus (Hotz and Bang, 1956). Although some of this virus may have been first absorbed onto the dynamic cell membrane and subsequently engulfed, in these vacuoles much of the virus remains free within the fluid droplet and yet is within the cell Pinocytosis is a general phenomenon among animal cells (Gey, 1955) and it seems likely that after the initial stages of virus adsorption, entrance into the cell is a part of this active cellular process. The electron microscope has so far not

* This summary includes work currently carried out by F B Bang at Johns Hopkins under a Research Grant from the National Microbiological Institute of the U S P H S.

dealing with infective virus, and I presume that in your material, Dr. Markham, the top component is non-infective.

Markham: Yes.

Williams: If you can separate infective and non-infective particles with a sufficient degree of precision

v. d. Ende: Of course, we do not know how much non-infective [illegible]

Burnet. It seems to me that the crucial question is how reliable is the technical method used in which ultracentrifugation is associated with sampling of the various levels. Has that method been applied to something which could be very accurately titrated, namely bacterial viruses, both in the form of pure virus and in mixtures of viruses of known size?

v. d. Ende: No, we have not done that.

Burnet: It seems to me that that would be desirable in this type of work.

[illegible]

[illegible]



FIG. 1 Thin section of macrophage in tissue culture engulfing virus particles during the process of pinocytosis

facing page 250

thrown any light on the immediately following stages, and we will therefore pass on to the later events in the cell.

The problem of the release of viruses of the *Myxovirus* genus from cells is intimately bound up with the nature of virus filaments, and it seems worth while to begin by considering the nature and properties of filaments.

Mosley and Wyckoff (1946) pointed out that the characteristic influenza filaments in the allantoic fluid often had spheres at their tips. In the first paper (Chu, Dawson and Elford, 1949) describing the frequent presence of filaments in recently adapted strains of influenza A virus, there is also a clear illustration of scattered dense spherical bodies along a less dense filament. The tissue culture work by Murphy, Karzon and Bang (1950) showed the same relationship—variable segmentation along the filament. Wyckoff (1953) reported it in sectioned material A recent examination of filamentous strains obtained directly from the allantoic fluid show again the same phenomenon—not common but unmistakably present (Figs. 2 and 3). Thus filaments may have spheres along their length. Since the filament originates from the cell the problem then is: How much of the filament is virus and how much is host cell?

To begin to answer this we must refer to some of the criteria for identification of the spherical forms as virus particles (Bang, 1955*b*).

Since these spheres

(1) are distinctive in appearance,
(2) are associated with infection,
(3) accompany the infectious unit when this is separated or concentrated by physical means,
(4) accompany the haemagglutinating activity of the virus,
(5) are agglutinated by convalescent sera,
(6) produce infection when very few individual particles are inoculated,

we identify them as virus particles.



Fig. 1 Thin section of macrophage in tissue culture engulfing ... les during the process of pinocytosis

facing page 250

[illegible]



Fig 4 Influenza A (Persia) filaments plus 1/100 dilution normal ferret serum

Fig 5 Influenza A (Persia) filaments plus 1/100 dilution of convalescent ferret serum Clumps like this one occurred in scattered areas of the screen Filaments are apparently lined up next to each other and spheres are adherent to the filaments



FIG 6 Thin section of allantoic cell lining allantoic sac—infected with influenza A (Persia—recently isolated) 48 hours This clump of filaments was studied in serial section and the continuity of the filaments with the cell surface demonstrated Other areas of the cell surface were free of filaments × 28,800.

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With the filaments the evidence is not so complete.

(1) They are distinctive in appearance.

(2) They are associated with infection, i.e no such *regular uniform long* filaments have been found in other infections, or in the absence of infection.

(3) They have not in themselves clearly been shown to be infectious.

(4) (a) They may be absorbed on red cells.
  (b) The long filament is broken down into pieces during absorption which indicates the presence of haemagglutinating activity all along the filament. (Experiments illustrating this in Figs. 2 and 3)
  (c) A further extension of this is the finding of Donald and Isaacs (1954) that ultrasonic treatment of the filament increased the haemagglutinating activity without a corresponding increase in infectivity.
  (d) Enzyme activity and toxicity are also greater in the filament than in the sphere (Edney, 1955).

(5) They may be agglutinated by convalescent sera (Chu, Dawson and Elford, 1949) (An experiment confirming this is shown in Figs. 4 and 5)

(6) Since no pure preparations of filaments have been made it has been difficult to show their infectiousness, and thus we cannot determine how many filaments are necessary to infect

The origin of the filament was suggested by the finding of short microvilli on the surface of the normal cells which are subject to infection (Murphy and Bang, 1952; Bang and Gey, 1952). Several authors have suggested that the filaments are extrusions from abnormal cells (Robinow, 1950; Hoyle, 1950, 1954). However, only with electron microscopy can one define the very long fairly regular nature of the characteristic filament of influenza. Thin sections of the allantoic cells infected with the filamentous influenza virus strains have not previously been prepared. Fig. 6

shows a section of a portion of a flat allantoic cell, showing the protrusion of many filaments. The cell membrane appears continuous with the membrane of the filaments. These filamentous spaghetti-like extrusions did not occur over the entire surface of the allantoic cells, but were spotty in distribution. Previous work on sections (Murphy and Bang, 1952, Wyckoff, 1953) was limited to infection with adapted or partially adapted strains, and such long filaments were not seen. Sections of the membrane infected with a well-adapted (Melbourne) strain showed no filaments (Fig. 7), but their occasional occurrence (Fig. 8) in the allantoic fluid of the same embryo indicates that either an exceptional cell or an occasional place on an infected cell yields such protrusions. Tissue cultures have also shown a profusion of filaments even when infected with the PR8 strain of influenza A (Murphy, Karzon and Bang, 1950) which normally does not produce filaments when grown in the chick embryo.

The fairly complete evidence that some virus activity occurs along the length, and thus presumably the surface of the filament, with the lack of evidence of multiple units of infectiousness, suggested that this form might correspond to the "incomplete" (von Magnus, 1951) or "non-infectious" form of the virus. However, it has been shown that the incomplete virus is spherical (Werner and Schlesinger, 1954), although like that of fowl plague (Schafer and Schramm, 1950) it collapses more readily on drying than does the "complete" virus. A comparison of complete and incomplete forms of the adapted Melbourne confirmed the above findings.* A comparison of a filamentous (Persia) strain showed similar numbers of filaments and spheres in the complete and incomplete form (Fig. 9). It appears, therefore, that the process of filament formation is independent of incomplete virus production. The filamentous form is manifestly inadequate in that segmenta-

* The "incomplete" virus preparations used in this work were prepared by making three successive passages of undiluted allantoic fluid at 24-hour intervals; they had infectivity ratios (i.e. ratio of infectivity/haemagglutinin titre) of approximately 0 001 relative to the corresponding "complete" virus preparations.



Fig. 7 Allantoic cell infected with influenza A (Melbourne) well adapted to growth in allantoic sac. × 20 000



FIG 8 Clump of filaments found in allantoic fluid from above embryo infected with Melbourne (well-adapted) Influenza A Most particles found elsewhere are spheres but an occasional filament is also found.

FIG 9 Filaments obtained from "incomplete" preparation of influenza A (Persia) recently isolated.



Fig. 10 Thin section of allantoic sac cells exposed to high concentrations of well-adapted (Melbourne) virus, necessary to produce incomplete preparation. Allantoic fluid from this embryo showed collapsed spheres

Fig. 11 Thin section of allantoic sac cells exposed to high concentration of filamentous (Persia) virus. Allantoic fluid from this embryo presented in Fig. 9.



FIG. 8. Clump of filaments found in allantoic fluid from above embryo infected with Melbourne (well adapted) Influenza A. Most particles found elsewhere are spheres but an occasional filament is also found.

FIG. 9. Filaments obtained from "incomplete" preparation of influenza A (Persia) recently isolated.

tion has not continued along its entire length. This may be due to a basic absence of infectious material along its length or merely to a lack of some factor which promotes division or segmentation. Presumably the adapted strain is one which has "learned" to use the host's material in one or the other of these ways.

The same embryos which furnished the "incomplete" virus studied in the allantoic fluid, were also studied by sectioning techniques. The large inoculum necessary to produce the "incomplete" virus in the case of the adapted Melbourne strain has destroyed most of the cell's architecture (Fig 10). The recently adapted strain Persia did not destroy the cells, but produced fairly numerous filaments along the allantoic surface. These filaments were shorter in the sections (Fig 11) but the degree of specimen selection in this study made it impossible to decide from the sections alone whether the length of the filament differs from complete to incomplete

Virus release from the cell has previously been studied in [illegible] ease virus [illegible] Wyckoff, [illegible] The data on the effect of Newcastle disease virus have been summarized (Bang, 1955a). A strain (Enders) of mumps virus, long adapted to life in the allantoic sac of the embryo was similarly studied Sections of the membrane 3, 4 and 5 days after infection were prepared. Only in the 5 day infection were there clear changes. Short ballooning microvilli appeared at the surface of intact epithelial cells (Fig 13) No distinctive morphological unit was seen within these extrusions We will refer later to sections of the pellet prepared by centrifuging the allantoic fluid to concentrate the virus (Fig 12)

The studies on fowl plague (Hotz and Schäfer, 1955) have been limited to the initial changes following large doses of [illegible]

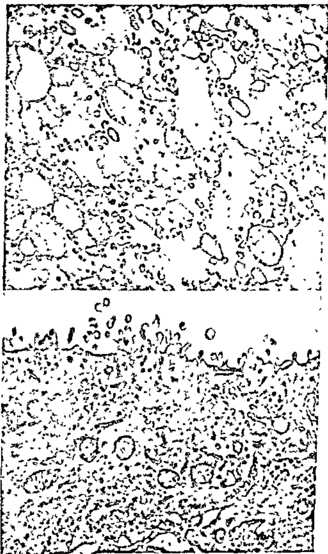
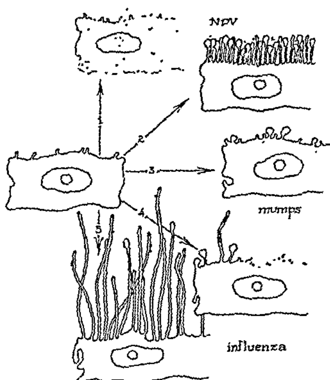


Fig. 12 Thin section of pellet of mumps virus obtained by centrifugation of virus harvested at 5 days. Mitochondria may be recognised as large balloons throughout. Smaller sacs with denser walls presumed to be virus. Oval appearance due to compression artifact.

facing page 253


NDV
1
2
3
4
5
mumps
influenza

emphasized that these diagrams are necessarily based on a combination of data, and it is not established that any one cell within the allantoic sac would show all these changes. Indeed it is likely that the occasional filaments occurring in an adapted strain are the product of particular cells, and a continued search of the membrane would be necessary to find them. The apparently haphazard method of extruding virus particles from cells appears to furnish an explanation for the variability in the diameter of the particles of the *Myxovirus* genus in contrast with the more uniform diameter found in particles of the smaller viruses.

Earlier we referred to our failure to demonstrate virus within the cell. A section of concentrated mumps virus, prepared from the same embryo which was studied in sections is shown in Fig 12. Although virus particles are present within this pellet, comparison with recent electron micrographs of a microsome fraction obtained from liver, or indeed with the pieces of endoplasmic reticulum seen in normal cells, shows a strikingly similar appearance and cautions us against a too hasty interpretation (Kuff, Hogeboom and Dalton, 1956).

Higher resolution pictures of the filaments of influenza show that some of the variation in thickness in sectioned material can be explained by the fact that one section includes but part of a wavy filament (Fig. 15), and yet emphasize the continuity of the cell membrane with the filament membrane. This is in agreement with the excellent micrographs of Morgan and Rose (unpublished). If the filaments of influenza are first fixed with osmium and then stained with phosphotungstic acid, not only is the membrane of the filament apparent, but variation in density of the internal material is clearly visible (Valentine, personal communication) (Fig. 16). This emphasizes the need for some analytical morphological study of the filament.

In conclusion, we believe that it is well established that the filament of influenza originates as an extrusion of a pathological cell, and that certain virus material is contained within

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In conclusion, we believe that it is well established that the filament of influenza originates as an extrusion of a pathological cell, and that certain virus material is contained within

it. It has not been established whether there are regular units of infectiousness along its course but the evidence is strong that haemagglutinating, and probably enzymatic, activity occur along its length. Since the membrane of the filament is continuous with the cell membrane, and since other members of the group seem to originate as a budding or pinching off of the cell membrane, opportunity for the incorporation of host protein is great (Knight, 1946; Smith, Belyavin and Sheffield, 1955).

Because of the great similarity of sectioned virus particles with microsome fractions of cells and with endoplasmic reticulum, we have been unable to identify virus members of this group within cells. Finally, the filament does not appear to be a homogeneous structure. Although most of the data in this paper have been limited to cells of the allantoic sac membrane, the fact that a profusion of microvilli occur on the surface of normal respiratory ciliated and non-ciliated cells, suggests that the filament cell-surface problem is a general one.

Acknowledgement

Figures 2, 3, 4, 5, 8, 9 and 16 were made by Mr. R. C. Valentine of the National Institute for Medical Research, to whom we are indebted for their use.

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DISCUSSION

Burnet. I think the host cell surface takes an essential part in the fabrication of influenza virus, and is naturally a point at which contamination, in one sense or another, of host material must occur

[illegible]

38) that fully infective virus only seems to come into existence as it passes through the cell surface. Also indicative of the importance of the cell surface is the presence in the influenza virus of two important components lipids, including all types characteristically found in the host cell, and polysaccharides, on which Gottschalk and Ada have recently carried out an investigation. They showed that the polysaccharides of the virus are very like those of the cell.

[illegible]

virus protein molecules carrying both enzymic and serological character. Within this surface membrane we have a collection of RNA which was, as it were, trapped by the formation of the sheath of the virus particle. This picture can be readily modified to cover the two other types. The

it. It has not been established whether there are regular units of infectiousness along its course but the evidence is strong that haemagglutinating, and probably enzymatic, activity occur along its length. Since the membrane of the filament is continuous with the cell membrane, and since other members of the group seem to originate as a budding or pinching off of the cell membrane, opportunity for the incorporation of host protein is great (Knight, 1946; Smith, Belyavin and Sheffield, 1955).

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Burnet. It is quite interesting and easy to examine filaments under a dark ground, a method which allows the use of [illegible]

[illegible]

molytic for fowl cells

Bang: I was hoping you would mention this work because I think we badly need some new and better method for analysing what is within this filament.

Some pictures just prepared by Mr. Valentine [illegible]

[illegible]

that the number is greatly diminished

Smith: I think that Burnet's very interesting suggestion may open up just a possible line of investigation. You can get some of these viruses [illegible]

[illegible]

centrifugation.

Bang You cannot for some [illegible]

filament certainly has a significant amount of functional virus protein on its surface and may represent a sort of space-enclosing change in the cell surface, which leads to the formation of an indefinitely extended filament. From Schafer's pictures of the incomplete form one might guess that this form is due to a partial failure of the reinforcement with virus protein giving rise to another type of space-enclosing shape.

Hirst: The only place in which Rose and Morgan can find the virus particles is on the outer surface of the cells and they appear there in very nice array and covered by a thick coat, but they also find them just beneath the cell surface, although not so well coated. I think it is clear that they are not formed by a virus fragment pushing through the membrane and then a bud of the cell membrane being pinched off. Inside the filaments Rose and Morgan also find some structure which they don't find inside the long filaments There is doubtless a pool of coating material just beneath the cell surface The production of long filaments may be analogous to the formation of silk fibres. A coated particle passing through the cell membrane pulls out a long thread of coating material after which it fails for some reason to get pinched off.

Williams Dr. Burnet has implied that in the electron microscopy of a virus, if one sees a differential opacity such as to make the interior of the virus, say, look photographically darker, this appearance is an indication of the presence of nucleic acid. Now, I do not really believe that, with respect to electron opacity, one can distinguish in a section of ordinary thickness between a material with the average density of nucleic acid and the average density of protein As an example of this, if one examines electron micrographs of unshadowed influenza virus, there is no evidence of any internal sort of structure. Any RNA that might be in the core simply does not show up.

The only evidence that I know of that might be conclusive in this connection is that in the case of bacteriophage if one stains these with ordinary osmic acid staining methods in an attempt to disclose the central part of the virus, one discloses not the centre but the periphery because of the osmophilia of the protein coat. It takes a specific staining for the nucleic acid to bring out the centre.

Dr. Bang, is there any reason to believe that if the filaments were examined in a state such that they had not been allowed to flatten, would they have the same diameter as the virus? Whenever I look at these pictures I get the impression that the filaments are flattened more than the virus and hence might have had an initial diameter considerably less than the virus.

Bang. Just before I came, I compared the pictures of sections with those of the dried filaments that you saw here today. They were taken with two different microscopes so it is a little hard to be sure that a comparison is valid. The filament in the section is a little thinner than that of the flattened one in the dried preparation. However, I think that that is partly due to the fact that I obviously got one section through one part of the virus and that the diameter varies. I do not have the impression that there was any major difference in size althou I simply don't have quantitative data to answer you.

Burnet: It is quite interesting and easy to examine filaments under a dark ground, a method which allows the use of wet reagents in a rather simple fashion. Some experiments I have done recently indicate that, with only one exception, anything which will haemolyse a chicken red cell will break down filaments, suggesting very strongly that the compounds in the surface of the filament are of the same general character as those of the surface of a cell.

The range is quite wide—it included hypotonic solutions, detergents, snake venom and lysocithin. Saponin was the one exception, a particu-

Bang: I was hoping you would mention this work because I think we badly need some new and better method for analysing what is within this filament.

that the

centrifugation.

Bang: You cannot for some peculiar reason get pure preparations; several people have tried, first Murphy, and then Isaacs and Donald. Dr

of an experiment?



FIG 1 (Williams) Electron photomicrograph of a shadowed preparation of PR8 influenza virus

Bang: No. We haven't had clean preparations, which is why we cannot say anything definite about the infectiousness

Burnet: Whether the appearance of these things in the dark ground is an optical illusion or not, certainly some of the filaments have more refractile points along their length which appear to me to equate very well with the knobs in the electron micrographs. You can count those which have knobs, and I have seen a few with more than one knob.

Hoyle: You often see them with more than one knob I think that many of these observations described by Dr. Bang can be made better by the dark field method than by the electron microscope method, because you can watch these protrusions emerge from the host cell, and can follow the sequence of events. The action of detergents on the filaments is very interesting. Almost all detergents break up the filaments, and also lyse red cells in very much the same concentration. But detergents are divided in their action on bacteria, for instance, very sharply into those which are highly bactericidal and those which are not bactericidal at all. The virus particles behave more like a host cell than like a bacterium, in that there is no difference in effect between anionic and cationic detergents

Isaacs: Dr. Bang mentioned that these filaments broke up very neatly, and it is very striking to examine the filaments directly: some of them are as long as 50 μ, which means that their length is 500 times their breadth. We have never been able to purify them, because we always get at least 20 per cent of spheres present, but with Prof Wilson Smith's suggestion, you could assume that the vast bulk of the preparation would be filamentous and you can separate the filaments out by filtration and get a preparation which is practically pure spheres, so that you can compare the two preparations, even although the filaments are never pure.

Williams. Some remarks have been made during the past few days concerning the possibility of a limiting membrane around influenza, and I have a slide which is certainly amusing and might bear on the point. Figure 1 (of rather poor quality) was taken by Wyckoff and myself almost 10 years ago and, at first glance, appears like a collection of sea-gulls As a matter of fact, it is a shadowed preparation of PR8 influenza virus, and much to our surprise we found that there is apparently a wing-like projection on each particle. If you note the character of the shadow of the "wing", compared to the character of the shadow of some relative sharp object like the one intact virus particle seen at the lower right, the edge of the shadow of the wing is rather fuzzy. This would indicate that the wing was created during the shadowing process, and was consequently shadowed only lightly The next thing to notice is that the long direction of the wing is at right angles to the shadowing direction We found this orientation universally to be so. The only conclusion that we were able to draw from this was that what looks like a wing is really in three-dimensions a fan coming out of virus particle; one would deduce this from the general length and character of the shadows. It appears as though during the shadowing the virus had been internally and differentially dehydrated or heated, and the virus popped. I think this is a strong indication that there is some membrane around the virus which

has a sufficient amount of rigidity that some degree of internal pressure is established before the membrane finally bursts and allows material to be spewed out. For whatever this evidence is worth, then, it would indicate that there is some type of mechanically integrated limiting membrane.

Stoker: With reference to other viruses, I think Dr. Coons has some-

[illegible]

think the whole development of the plaques in Dulbecco's technique indicates spread of virus from cell to cell, rather than out in the fluid Melnick has also described the presence of secondary micro-colonies spreading through the fluid as secondary, distinct plaques, whereas the

[illegible]

Bang It was about 5 times as thick, but it was made up of a series of individual ones that were of exactly the same size as the typical filament. Thus it was like a rope.

[illegible]

INTERACTION OF PHAGES WITH BACTERIAL CELL WALLS AND THE DEVELOPMENT OF PHAGE IN THE WALL-LESS PROTOPLAST

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THE sequence of events following the collision of a bacteriophage particle with a susceptible bacterial cell is now fairly well understood. The initial interaction involving the adsorption of the phage on to the cell surface occurs in two stages—a reversible step during which time the phages may be recovered from the surface by transferring into a medium that does not favour adsorption, followed by an irreversible step (Puck, Garen and Cline, 1951, Stent and Wollman, 1952). The factors effecting the reversible attachment to the bacterial surface have been found to be identical to those required for reversible adsorption of phage onto "chemically defined" surfaces, such as cation exchange resins and glass (Puck, Garen and Cline, 1951) However, it would seem that the irreversible attachment and subsequent injection of phage DNA into the host cell involves processes of a more circumscribed nature. The specificity of bacteriophage action would appear to be governed by the presence of characteristic mosaics of molecules in the cell surface. With our increasing knowledge of the nature of the bacterial surface, it is not surprising to find that some of the superficial structures provide the bacteriophage with receptor systems.

The Location and Chemical Constitution of Bacteriophage Receptors

them, I am quite certain.

Hoyle: I think, actually if you watch from the beginning they always have a knob on the end at the start, but that quite often the knob breaks off into a sphere, and leaves you the filament without a knob on the end.

interaction between the receptor sphere and T5 phage particles has been elegantly demonstrated in the electron microscopic study by Weidel and Kellenberger (1955).

Isolated cell walls of several other bacterial species have been shown to react with phages specific for the organisms.

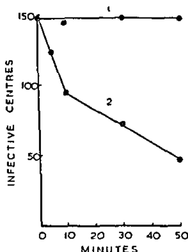


FIG. 1 Interaction of *B megaterium* phage C with isolated cell walls of *B megaterium* (150 μg. dry weight/ml) suspended in 5 per cent peptone at 37° C. Curve 1—cell walls were digested with lysozyme (5 μg /ml) for 30 min at 37° C before the addition of the phage suspension. Curve 2—showing phage inactivation on incubation with cell walls (From Salton, 1956b).

Hotchin, Dawson and Elford (1952) demonstrated the adsorption of staphylococcus K phage by the isolated "cell membranes or shells" of the host *Staphylococcus aureus*. Salton and Stent (unpublished observations) found that the phage C for *Bacillus megaterium* was inactivated by isolated walls of this organism. The latter system seemed to be an

the receptors studied by Miller and Goebel (1949) belong to the surface components possessing marked antigenic properties (somatic antigens), components that do not appear to be essential for the maintenance of the cell's morphological integrity. In isolating the receptor substances from *Escherichia coli* B, Weidel (1950) discovered that he had isolated the cell walls of this organism! Thus the phage receptors were shown to be an integral part of the rigid cell-wall structure.

The purified antigen isolated from Phase II *Shigella sonnei* by Jesaitis and Goebel (1952) was found to be a protein-lipocarbohydrate complex. All of the T phages to which the organism was susceptible were inactivated by the complex, and the antiviral properties were confined to the lipocarbohydrate part. The latter contained 29·8 per cent lipid, and the monosaccharide components were identified as glucose, galactose, glucosamine and an aldoheptose. It is of interest to note that the ability to inactivate T4 phage was lost on extracting the lipocarbohydrate with 70 per cent ethanol; antiviral activity could be restored by adding the lipid extract to the treated complex, but this restoration was not specific for the bacterial lipid as other saturated fatty acids behaved similarly (Jesaitis and Goebel, 1953).

Weidel (1951) showed that the isolated walls of *E. coli* B possessing phage receptor activities, were of lipoprotein nature. Subsequent investigations with soluble preparations of receptors for T3, T4 and T7 established that they were complexes of lipid-carbohydrate and protein, the lipid contents being of the order of 30 per cent (Weidel, Koch and Lohss, 1954). The monosaccharide constituents have been identified as glucose and glucosamine and the presence of a heptose [illegible] demonstrated (Weidel, [illegible] B and *Shigella spp.* have [illegible] its in common (Weidel, 1955).

The T5 phage receptor possesses a similar constitution to those for the other phages, and the isolated receptor substance has been shown to be in the form of a spherical particle. The

approximately 50 per cent polysaccharide (as determined by reducing values after acid hydrolysis) and 50 per cent polypeptide. One interesting feature of the constitution of these

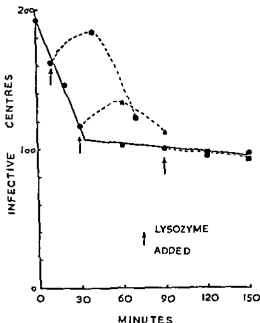


FIG. 2. The effect of lysozyme on the course of inactiva-

broken line curves

walls is the limited range of amino acid components. Diaminopimelic acid, alanine and glutamic acid are the major amino acid constituents of *B. megaterium* cell walls (Salton, 1956*a*). *M. lysodeikticus* walls possess alanine and glutamic acid in common with *B. megaterium*, but contain in addition

attractive one for further investigations owing to the susceptibility of the walls of this organism to digestion by lysozyme (Salton, 1953).

The inactivation of phage C by isolated cell walls of *B. megaterium* is illustrated in Fig. 1. If the walls are digested by lysozyme before the addition of the phage suspension, no inactivation occurs. Such a destruction of receptor activity was not surprising, as Pirie (1940) had demonstrated the inability of heat-killed, lysozyme-treated cells of *B. megaterium* to adsorb phages. The addition of lysozyme at various times after mixing bacteriophages with cell-wall suspensions, results in a "temporary" release of phage as shown in Fig. 2. The proportion of inactivated phages is unaltered after about 90 minutes due to the dissolution of the residual cell-wall structure. The addition of lysozyme at times earlier than those shown in Fig. 2 appears to stop the subsequent decline which occurred when lysozyme was added after 10 and 30 minutes exposure to the walls; for example, when lysozyme was added after 3 minutes from the time of mixing phages and walls, *there was no further change in the number of infective particles* for up to 60 minutes. The "temporary" release of phage on the addition of lysozyme at 10 and 30 minutes could be due to "desorption" on dilution of the suspension at the time of sampling.

Professor Naylor has kindly provided me with several phages for a strain of *Micrococcus lysodeikticus* and the interaction of his N4 phage with isolated cell walls of the host strain shows a similar course of inactivation as that observed [illegible]

As "receptor substances" the cell walls of *B. megaterium* and *M. lysodeikticus* differ in composition from the *E. coli* cell wall and isolated receptors. Unlike the *E. coli* substances, the walls of these lysozyme-sensitive organisms contain little or no lipid material. The walls of *B. megaterium* and *M. lysodeikticus* are of mucocomplex nature, being composed of

walls are not completely degraded by the enzyme, lysozyme, for there is an appreciable liberation of reducing substances when the "soluble wall material" is incubated with lysozyme. However, preparations have lost their antiviral activities for their appropriate phages. Neither bacteriophage inactivation nor lysozyme digestion is blocked when *B. megaterium* is treated with 1-fluoro-2:4-dinitrobenzene (FDNB) Unpublished experiments performed by Dr. Ingram and the writer have shown that treatment of the walls of this organism with FDNB give DNP-alanine as an end-group amino acid as well as mono-DNP-diaminopimelic acid.

A complex mixture of fragments of varying molecular sizes results from the digestion of the cell walls of *B megaterium* and *M. lysodeikticus* with lysozyme (Salton, 1956*a*, *b*) The loss of phage receptor activity on dissolution of the walls with lysozyme is not difficult to understand since the spatial relationships which such fragments must have shared in the intact walls is replaced with a disorderly array of molecules in solution. It is hoped that further studies may reveal the nature of the cell-wall mosaic reacting with the phages and the location of such a mosaic in terms of the linkages attacked by lysozyme. The disintegration of cell walls catalysed by adsorbed phages has been reported by Weidel (1951), and it would be of considerable interest to determine the nature of fragments that may be released from the walls of these lysozyme-sensitive bacteria after reaction with their specific phages.

Bacteriophage Multiplication in Protoplasts

The sensitivity of certain bacteria to lysozyme has presented us with a unique opportunity of studying some of the relationships between structure and function in the bacterial cell. Weibull (1953) has shown that treatment of *B. megaterium* with lysozyme in the presence of sucrose (0·15 M) results

glycine and lysine. Glucose, glucosamine and an unknown substance giving the reactions of an amino sugar are found in both cell walls. An acetylamino sugar complex (composed of

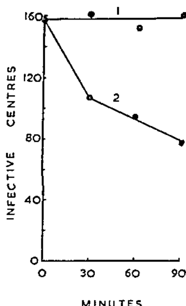


FIG. 3. Interaction of N4 phage with cell walls of *M lysodeikticus* (200 μg dry weight/ml.) suspended in tryptone broth at 37° C. Curve 1—cell walls digested with 5 μg. lysozyme/ml for 30 min at 37° C before the addition of the phage suspension Curve 2—showing inactivation of the phage by incubation with cell walls

glucosamine and the unknown amino sugar reacting substance) is liberated from cell walls of both organisms on digestion with lysozyme (Salton, 1956*a*).

att

the ..

common linkage attacked by lysozyme, there is no cross

McQuillen, 1955), but this was perhaps not surprising in view of the complex physiological conditions governing "aptitude" (Lwoff, 1953).

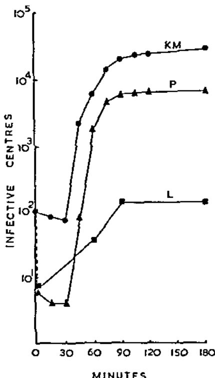


FIG 4 Bacteriophage multiplication in *B megaterium* KM KM—intact cells; P—protoplasts, L—lysed protoplasts Cells were infected prior to protoplast formation as described by Salton and McQuillen (1955)

So far, bacteriophage multiplication has been demonstrated in protoplasts of two lysozyme-sensitive bacteria—*B. megaterium* (Brenner and Stent, 1955; Salton and McQuillen,

in the dissolution of the cell wall, leaving the inner cellular structures in the form of a relatively stable spherical protoplast. Recent investigations have shown that protoplasts of *B. megaterium* retain much of the structural organization of the bacterial cell and that many of the synthetic processes, such as protein and nucleic acid formation, are not seriously impaired by the absence of an outer cell-wall structure (McQuillen, 1956; Weibull, 1956). Although the bacterial protoplast can grow and appears to undergo a process of division (McQuillen, 1956), it does not revert to the rod-shaped organism nor does it give rise to colonies when studied under the normal conditions used for cultivating the original organism.

It was of interest, then, to determine whether the bacterial protoplast which was unable to revert to the normal cell could support the production of bacteriophages, by removing the cell wall with lysozyme after the initial infection process had been allowed to take place. Several independent investigations have confirmed the ability of protoplasts to support phage multiplication (Brenner and Stent, 1955; Mutsaars, 1955; Salton and McQuillen, 1955). Such results are in agreement with the present concepts of bacteriophage infection, illustrating the dispensability of the wall, once the release of phage DNA has been "triggered" by the receptor mechanisms. These investigations also show that the protoplast membrane must be a structure of sufficient flexibility to allow the entry of phage DNA without seriously damaging its functions of [illegible] o support the [illegible] by the data from Salton and McQuillen (1955) presented in Fig. 4. Studies with the lysogenic *B. megaterium* 899 have shown that cells induced with hydrogen peroxide prior to removal of the wall with lysozyme can subsequently produce new phage. The growth of phages in protoplasts of the lysogenic *B. megaterium* is illustrated in Fig. 5. Attempts to bring about a direct induction of protoplasts were unsuccessful (Salton and

McQuillen, 1955), but this was perhaps not surprising in view of the complex physiological conditions governing "aptitude" (Lwoff, 1953).

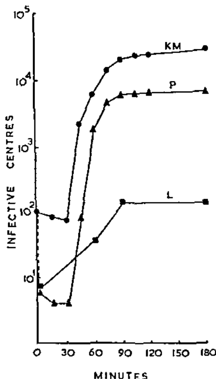


FIG 4. Bacteriophage multiplication in *B megaterium* KM. KM—intact cells, P—protoplasts; L—lysed protoplasts Cells were infected prior to protoplast formation as described by Salton and McQuillen (1955)

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The ability of protoplasts of *B. megaterium* to support the multiplication of virulent phages is illustrated by the data from Salton and McQuillen (1955) presented in Fig. 4. Studies with the lysogenic *B. megaterium* 899 have shown that cells induced with hydrogen peroxide prior to removal of the wall with lysozyme can subsequently produce new phage. The growth of phages in protoplasts of the lysogenic *B. megaterium* is illustrated in Fig. 5. Attempts to bring about a direct induction of protoplasts were unsuccessful (Salton and

One of the central problems in mind, during these studies attempting to integrate structure and function, has been the possibility of determining whether the bacterial cell possesses any potentialities for synthesizing bacteriophages other than those circumscribed in their infectivity by the presence of a specific receptor. The answer to such a problem would enable us to decide whether multiplication of phages in a given host is governed solely by a receptor system that effects the entry of phage DNA or whether bacteriophage growth is governed also by "intraprotoplast" factors. The immunity of lysogenic organisms to their own phages (Lwoff, 1953) would seem to be governed by intracellular factors or events. Bacterial protoplasts may offer one possible experimental system of testing the ability of the protoplast to synthesize an "unrelated" phage; this could be tested by coating the protoplast surface with a "soluble receptor" specific for the "unrelated" phage. In this way it may be possible to find out if protoplasts of *B. megaterium* provided with soluble receptor for *M lysodeikticus* phage could support the growth of the *M. lysodeikticus* phage and to test the converse system with soluble receptor for *B megaterium* phage on protoplasts of *M. lysodeikticus*. Unfortunately, the soluble wall material from these organisms has lost its receptor activity, and until some method of retaining receptor activity is found we shall not be able to answer the problem with this protoplast system.

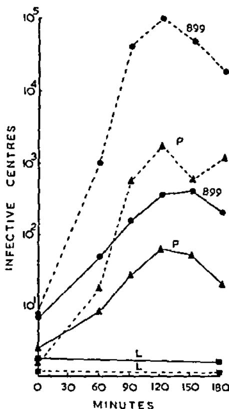
Acknowledgements

I should like to thank Professor Naylor for supplying me with the bacteriophage and host strain of *M. lysodeikticus* prior to the publication of their isolation and description.

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1955) and *B. subtilis* (Mutsaars, 1955). Recent experiments have also shown that protoplasts of *M. lysodeikticus* can



support the development of phage, but owing to the fragility of these protoplasts the results have been less dramatic than those observed for *B. megaterium*.

causes it to burst holds completely, because I would not think that the protoplast membrane would be nearly as tough as the cell wall of the bacterium.

Salton: No, I think it is certainly much more fragile a structure. That was shown by the so-called eclipse period, on dilution of protoplast suspensions for phage assay, the dilution of the sucrose stabilizer [illegible]

[illegible]

acid bacteria

The cell walls themselves are not sensitive to trypsin. So it is conceivable that you might be able to degrade some fractions with other enzymes.

[illegible]

McQUILLEN, K. (1956) Bacterial Anatomy, ed. E. T. C. Spooner and B. A. D. Stocker, p 127. Cambridge University Press.
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DISCUSSION

[illegible]: Dr. Salton, have you any idea why the development of phage

causes it to burst holds completely, because I would not think that the protoplast membrane would be nearly as tough as the cell wall of the bacterium.

Salton. No, I think it is certainly much more fragile a structure That was shown by the so-called eclipse period, on dilution of protoplast suspensions for phage assay, the dilution of the sucrose stabilizer

Salton: No. Induction happens all right with the intact cells in sucrose medium. All these experiments were performed with the same

that some cell walls, at least, may contain the D-isomer of certain amino acids There is certainly D-glutamic acid in both of these, and possibly D-alanine D-Alanine has been found in cell walls of some of the lactic acid bacteria

The cell walls themselves are not sensitive to trypsin. So it is conceivable that you might be able to degrade some fractions with other enzymes.

Cook. What was your average chain length?

MCQUILLEN, K. (1956). Bacterial Anatomy, ed. E. T. C. Spooner and B. A. D Stocker, p. 127. Cambridge University Press.
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[illegible]
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DISCUSSION

[illegible]

[illegible]

weight of the organism, is completely digestive by lysozyme, so that [illegible]

GENERAL DISCUSSION

Burnet: In attempting to sort out the material that has been discussed and to say something in broad strokes of present day trends, limitations, opportunities and objectives in this field of physics and chemistry in relationship to viruses, I shall have to look at them very much from the point of view of a biologist's impression of what can be gained by the physiochemical approach with which we have been mostly concerned.

From this biological angle, there are two points which I think are essential, as much for people concerned with the physics and chemistry of viruses as for the biologist. First, whatever the host and the virus that are being considered, they represent a surviving system, whether it is a host-virus relationship in the real world of infectious disease, or in the laboratory world, where we deal, or tend to deal, with relatively artificial systems as, for instance, a well-adapted influenza virus and the chick embryo

Second, every infection by a virus represents the interaction of [illegible]

hood of successful transfer—the second through its capacity to [illegible]

disease of human beings or of organisms important to human beings I should like to mention first some of the directions in which academic work of the type that we have been [illegible]

[illegible]

anything that we can do in the matter of purification of viruses may help in the production of vaccine material, and anything that we might do in sorting out from crude virus material immunizing agents with the requisite qualities is equally desirable. Secondly, if we continue to use living vaccines, as we are now and may do more in

Salton: I don't know that Brenner specifically showed that DNA was released. I think he did try with some DNA preparation, isolated from the phage, and incubated that with protoplasts, but again nothing happened.

[illegible]

species but were unable to find any. So the negative experiments are devoid of significance.

Burnet Is that a generalization?

Lwoff No, it is not I think one should be able to obtain the passage of the DNA of the phage into a bacterium, but one has to find the right system.

Salton. Perhaps coating the soluble receptors on the protoplasts of another organism would be one way of getting it in. We still have hopes that we may be able to do this.

Lwoff: It is a very important experiment from a theoretical point of view because it is the only way to prove that a phage can be produced from pure nucleic acid.

Gard: Could you not, by use of micromanipulators, put a naked protoplast into any empty shell?

Salton Wámoscher dissected bacteria with micromanipulators, but no one has attempted that with protoplasts.

that we have a rather small amount of RNA in these viruses and we have some fairly complicated structures which require to be determined by the information contained in that RNA

In particular, it is clearly desirable to know what is the relationship between the protein associated with the nucleic acid in the soluble antigen (the bound antigen of Schafer) and the protein which is responsible for the specific viral characters of the surface of the particle, such as the serological surface character and the enzymic adsorptive activity. We must bear in mind the possibility that those two proteins may differ only because they are associated with other material: in the case of the specific soluble substance, with ribonucleic acid, in the case of the surface, with lipoid and, presumably, polysaccharide. I might interpolate that it is still not proven that the polysaccharide, which all the workers find in these viruses, is wholly a host component I should guess that it was, but the possibility that it is part of the antigenic pattern of the virus must still be faced.

[illegible]

handling virulence by genetic methods in animal viruses, and there are hints from Best's work in Australia that this may not be out of the question with regard to plant viruses The chemical work in Berkeley again suggests that eventually we may have a genetic approach to the understanding of virulence Further, with the development by Dulbecco of a much more refined and delicate analysis [illegible]

may have a means of tackling what I believe is the most fundamental question of all: In what does the virulence of a virus consist? What is this relationship between replication in a cell and cytopathogenic

the future, then it is obviously one of the major tasks of virology to produce the correct strains for such purposes.

We have been concerned with a strictly academic approach to the problem, and it has become rather evident in the course of this discussion that there are two main aspects from which we have approched this problem. On the one hand we have broad physical descriptions of structures, varying in size from the morphological to the macromolecular; two particularly good examples of such discussion were provided by Williams, at the electron microscopic level, and Dr Franklin, at the macromolecular level But I would stress that [illegible]

broadest terms, this second approach could probably be described as being concerned with recognizing and classifying the amount and type of information, in the modern sense, encoded in the macromolecules which make up both the virus particles and the host cells which they infect

Perhaps the two major objectives that such work will aim at will be, first, to exploit to the utmost what help work with viruses, in any particular field, can give to the understanding of protein synthesis, or perhaps more broadly to the general problem of the replication of specific pattern in organic macromolecules. The special virtue of virus studies is that they provide relatively self-contained systems in which no other component appears to be present than protein and RNA. This is particularly true for TMV. The second major objective is the analysis of the nature of virulence, and I should just [illegible]

following infections in single cells, Dulbecco's tissue cultures would be very much better than leaves of plants

Dulbecco. The difficulty is to get the right nucleic acid for the injection technique.

Lwoff The other difficulty is that if you have a concentrated preparation of nucleic acid, it will be very viscous, and I wonder whether it could be injected

Gard· With relation to what Bawden has just said, I would like

infection. It seems that some new aspects of the mechanism of infection and multiplication of the virus may come out of these studies I am not familiar with his technique, but according to the photographs that have been published of his work, it seems to be a technique that is reproducible and not just dependent upon happy circumstances and which can be used as a more or less reliable routine method.

Isaacs. One very interesting subject that has come up has been

plants and insects all seem to be unstable and to occur in small quantities, so that it will be very difficult to get them in a state suitable for any critical work on their constitution. As the strain of

also be affected. But surely many changes, including those not easily reversible, could happen without the need to go as far as turning

effect on that cell, and the various pathological effects which follow at higher levels?

*As a final remark, I would mention that it would be both un*realistic and unwise not to recognize that advance in this field, or any other field of science, cannot be forecast logically, but will depend on the emergence of the various factors which from time to time will allow a new break; particularly new ideas of technique, application of techniques from one field to another, the appearance of some unrecognized laboratory phenomenon, or the happy result of a fool experiment I feel that perhaps Dr Salton's suggestion of the possibility of infecting a protoplast by some trick of inducing the DNA of a virus to come into the protoplast, is just that sort of new technical idea which, although it may be a complete washout, yet has in it the potentiality of a really new field But even without the emergence of any outstanding novelty the elaboration and development of the sort of thing that we have been discussing should make it quite certain that there is a reasonably exciting time in front of us

Andrewes Gard, in discussing what Salton said, mentioned the prospect of micromanipulation, and Burnet has just told us that

the nucleic acid directly into cells by means of micromanipulation. That should be technically possible, but I should like to know from Bawden, as regards plant viruses, how far one could get if one could inject nucleic acid into a single cell; could you follow what happened?

Dulbecco. I think that if we could inject it, we could follow what

Bang· I haven't seen any recent pictures of APC's but certainly one suspects that that is what is happening

Williams· Is the type of nucleic acid known for herpes simplex?

Bang· I don't see how one could tell because no one has had a sufficiently clean preparation

[illegible]

Bang· How would he tell, except by looking at the inclusion, which would have all sorts of material?

Burnet I think it was the semi-purified virus

[illegible]

Because if we are going to encounter a lot of viruses which have got RNA *and* DNA in them, and that will rather confuse the issue

Bawden· Burnet has stressed the need for uniform lines of cells in studying viruses. The value of this has long been obvious with plant viruses, where we have worked with clonal lines of plants such as potatoes. The reaction of different clones to one and the same virus strain can differ enormously: one clonal variety may die when infected with a strain of virus X, which produces only a mild mosaic in a second clone and is carried symptomlessly by a third, although the virus may multiply more extensively in the second and third varieties. Virulence is not an intrinsic character of a virus, but is a measure of the way the virus and a given genotype of host react Whether a virus is harmless or lethal seems sometimes to be determined by a single gene in the host

I would add a third point to the two stressed by Burnet It is the need also to control the conditions in which the infected organism is kept A virus may multiply extensively in a clonal variety in two widely different environments, and in one it may cause a lethal disease and in the other be benign Differences between the conditions in which infected plants have been studied are perhaps responsible for [illegible]

[illegible] Burnet was suggesting about Puck's work

I gathered from Dulbecco that not much had come out yet in the

DNA, normally multiply in actively dividing cells. There is probably nothing to the idea, and I don't know whether the viruses that con-

late mature cells to divide.

Crick One of the experiments one would like to see is the multiplication of an RNA virus in a cell in which one has destroyed all the DNA. It is possible in some cells to destroy the DNA and allow protein synthesis to go on. If it could be shown that, without any DNA there, you could get replication of an RNA virus, this would be an important experimental result.

Dulbecco· Perhaps with some enucleated cell.

Smith· I should like to refer again to the question of the very great importance of the homogeneity or non-homogeneity of the virus produced in the cell, because it seems to me that offers a very important line of work. If the virus produced within a cell is not homogeneous and if you can separate off different sorts, one has got to account for that non-homogeneity. When does it occur?

As I showed yesterday, we have been able to separate off in pure form, one form, at any rate, quite distinct from another I do not suppose for a moment that that is the only example of non-homo-

Pirie· Bawden and I had a shot at that by giving plants radio-

so-called incomplete forms and degraded forms.

Williams: There is good demonstration that for one of the animal viruses the beginning of the growth is in the nucleus—I was thinking of herpes simplex. Do you know if this is true of any other viruses?

Bang: I haven't seen any recent pictures of APC's but certainly one suspects that that is what is happening

Williams: Is the type of nucleic acid known for herpes simplex?

Bang: I don't see how one could tell because no one has had a sufficiently clean preparation.

Williams: Here seems to be a virus that forms in the nucleus; it [illegible] both DNA and [illegible] told me this on [illegible]

Bang: How would he tell, except by looking at the inclusion, which would have all sorts of material?

Burnet: I think it was the semi-purified virus.

[illegible]

potatoes. The reaction of different clones to one and the same virus strain can differ enormously one clonal variety may die when in- [illegible]

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[illegible] gathered from Dulbecco that not much had come out yet in the

way of finding varying susceptibility of clones of cells from mixed populations, at least in Puck's case.

Dulbecco: We have no experience in this field, but I know that Puck looked for cells resistant to Newcastle disease virus in sensitive populations, and he actually has obtained clones of relatively resistant cells These cells seem not to be entirely resistant, but they certainly are considerably more resistant than the original population.

Stoker: Is the suggestion that HeLa cells were resistant to polio

It has not been derived from a clone but certainly from a uniform group of cells that had been in culture for more than ten years. The tumour cell is highly susceptible to the virus of encephalitis, whereas the normal derivative is resistant to the virus (Bang, F. B., and Gey, G. O (1952). *Johns Hopk. Hosp. Bull.*, 91, 427).

Williams: Puck is able to grow cells and so treat them that he gets non-dividing cells over a millimetre in diameter This over-

these are negative; nobody has looked very hard . but there seem to
[illegible] to these
[illegible] Yeast
[illegible] rosomes
[illegible] It also
contains DNA.

Lwoff Many scientists whom I know have looked for viruses in yeasts and were unable to find any.

* * * * *

Harington This brings us to the close of this meeting, and to my closing remarks It would be preposterous for me to try to make any scientific contribution to a summary of the proceedings of this conference; in any case, Burnet has done that with authority and to admiration. All I can possibly offer would be a sort of general impression I have received sitting here as an observer.

In my opening remarks, I did make the obvious statement that we have now reached the stage at which biochemistry and physics have clearly come into their own in virus research It did not take long for that to be illustrated, because on the very first day with the theoretical contribution of Dr. Crick and Dr Watson, the very beautiful papers read by Dr. Williams and Dr Franklin, and on chemistry by Dr. Knight, we had the best possible demonstration of the great power of both physical and chemical methods as they are now being applied I was, however, left at the end of that day with a little uncomfortable feeling, which I think perhaps Burnet shared, that the very power of those methods when applied to the simpler plant viruses had its own danger, in that it might disguise from people who were approaching the problem from the purely physicochemical point of view the greater complications that have to be dealt with in the case of animal viruses, where you have more complexity in the structures of the viruses themselves and in the host-virus relationship

I must say that I felt that [illegible]

meeting and I was very glad to find that Burnet also seemed to be feeling that we were reaching a stage in which some closer contact between the two sides was being achieved. That seemed to me to be reflected in the broadening of the discussions of the later papers, where there have been significant contributions to the discussion

both from the biologists and from the physicists and chemists. If, in fact, I am right in this conclusion, I think we may say that one principal object of the symposium has been achieved, namely that [illegible]

pleasant.

AUTHOR INDEX TO PAPERS

SUBJECT INDEX

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